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Use of Isotopically Chiral [4′-^{13}C]Famciclovir and ^{13}C NMR To Identify the Chiral Monoacetylated Intermediates in the Conversion of Famciclovir to Penciclovir by Human Intestinal Wall Extract

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ABSTRACT Famciclovir is the oral form of the potent antiherpesvirus agent, penciclovir. Hydrolysis of one of the acetyl ester groups of famciclovir creates a chiral centre leading to the possible formation of (*R*)- and (*S*)-enantiomers. During its conversion to penciclovir, famciclovir forms two chiral metabolites, namely monoacetyl-6-deoxy-penciclovir and monoacetyl-penciclovir. The absolute configuration and stereospecificity of the monoacetyl metabolites of famciclovir, produced in human intestinal wall extract, were determined using isotopically chiral famciclovir and ^{13}C NMR spectroscopy of the isolated metabolites. ^{13}C NMR showed that the esterase(s), in human intestinal wall extract, hydrolysed the acetyl group preferentially from the pro-(*S*)-acetoxymethyl group of famciclovir. The specificity of esterase action in forming monoacetyl-6-deoxy-penciclovir and monoacetyl-penciclovir was about 77 and 72%, respectively.

KEY WORDS: Chiral stereospecificity, esterase, metabolism, human tissue

Famciclovir (FCV, Fig. 1) is the oral form of penciclovir, a highly selective antiherpesvirus agent. Penciclovir [PCV, 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine, BRL 39123] is active against members of the herpesvirus family. In cell culture, penciclovir inhibited clinical isolates of herpes simplex viruses types 1 and 2 (HSV-1, HSV-2) and varicella-zoster virus (VZV).[1] Like acyclovir, penciclovir has highly selective activity against the herpesviruses because it is phosphorylated, and hence activated, only in herpesvirus infected cells.[2,3] However, as for other compounds such as acyclovir, penciclovir is poorly absorbed when given orally to rodents and man.[4,5] Famciclovir gave much improved blood levels of penciclovir in rodents and was converted to penciclovir by human tissue extracts[6] and so famciclovir was selected for further progression. Both famciclovir and penciclovir are being evaluated in clinical studies.

Famciclovir can form various intermediates during its conversion into penciclovir (Fig. 2). The two chiral metabolites of famciclovir, monoacetyl-6-deoxy-penciclovir (BRL 43594) and monoacetyl-penciclovir (BRL 42222), are formed when one of the acetyl ester groups is hydrolysed. In extracts of the intestinal wall from both human and rat tissues, the rate of hydrolysis of the first ester group was much faster than that of the remaining ester.[6] Indeed, after incubation for 240 min in an extract of human intestinal wall, the only major metabolite was monoacetyl-6-deoxy-penciclovir and monoacetyl-penciclovir was one of the minor metabolites. After oral administration of famciclovir to man,[7] unchanged famciclovir was not detected in plasma, but its monoacetyl derivative was found, though only at the first sampling time, 0.25 h after dosing. Monoacetyl-penciclovir was detected in an occasional urine sample. Since the monoesters are chiral compounds, it is possible that one isomer is formed preferentially. In order to determine the stereospecificity and absolute configuration of the monoacetyl metabolites, isotopically chiral [4′-^{13}C]famciclovir was synthesised with ^{13}C incorporation in one of the acetoxymethyl groups (**1a**)[8] and incubated with an extract of human intestinal wall. Subsequent ^{13}C NMR analysis of the metabolites isolated by HPLC was used to determine whether the remaining acetyl group was adjacent to the ^{13}C and thereby establish the specificity of hydrolysis of famciclovir.

Part of this work has been presented in a preliminary form as a poster at the International Conference for Anti-viral Research, Brussels, 1990.[9]

MATERIALS AND METHODS

Preparation of [4′-^{13}C]Famciclovir

[4′-^{13}C]Famciclovir was synthesized as described previously by Sime et al.[8] For ease of handling during synthesis, compound **1a**, enantiomeric purity >99%, was prepared with unlabelled compound **1** by adding an equal amount of unlabelled intermediate during synthesis. The enantiomeric ratios for preparations of the side chain intermediate were determined by HPLC on a Resolvosil-BSA column.[8]

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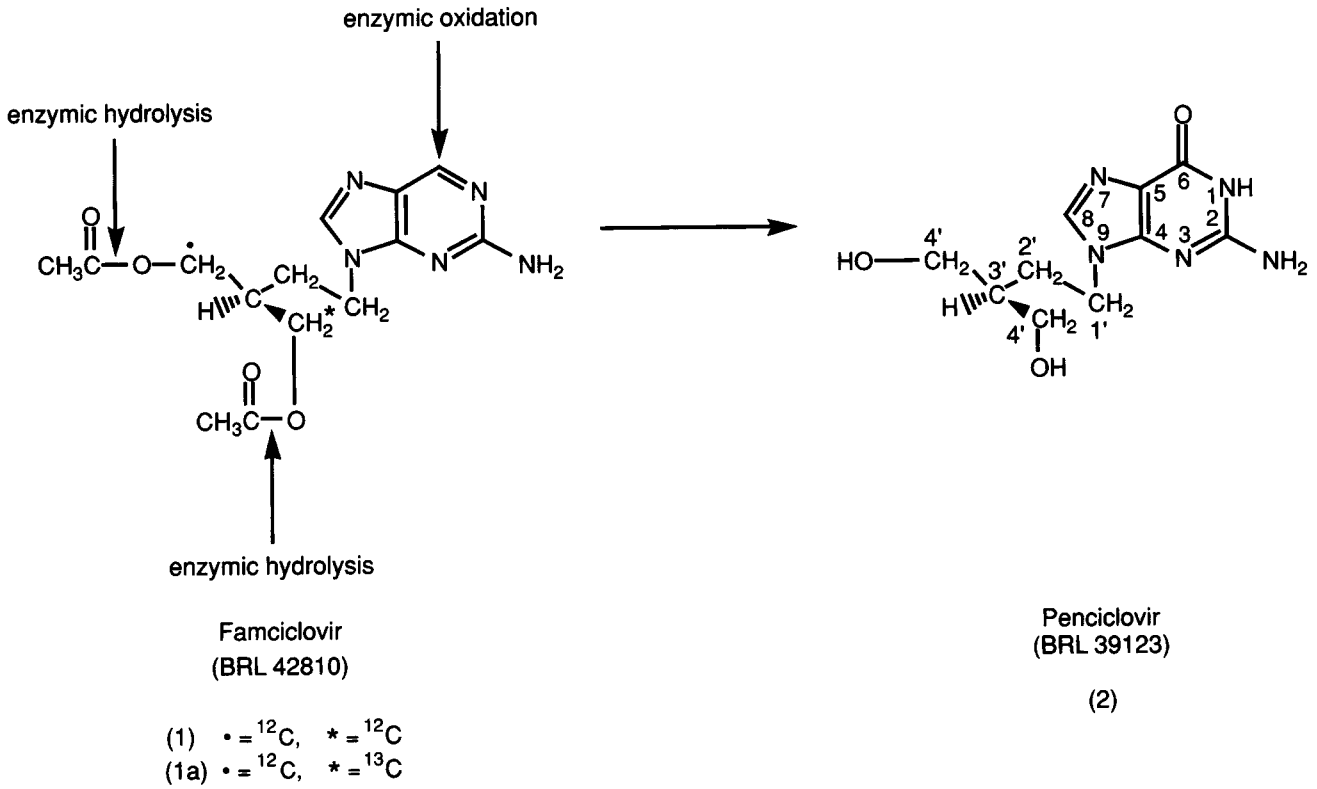

Fig. 1. Structures, and enzymic reactions required for the conversion of famciclovir to penciclovir.

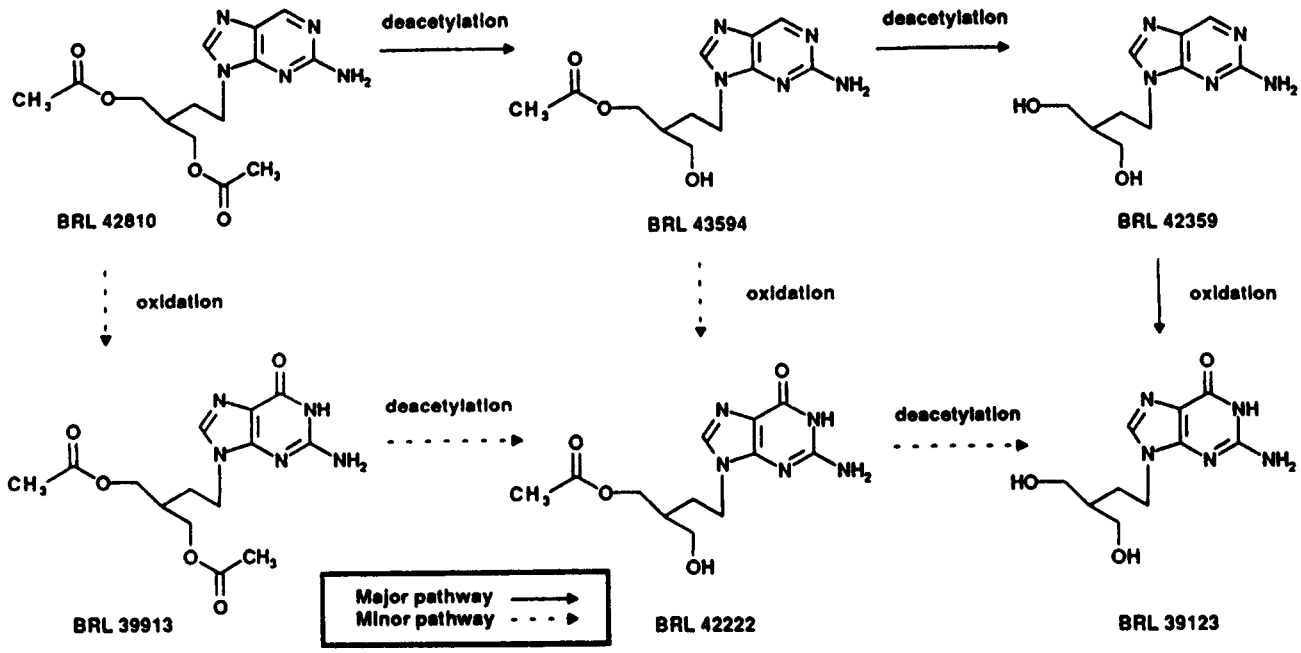

Fig. 2. Biotransformation of famciclovir in rat and human tissues.

Formation of Famciclovir Metabolites and Their Isolation

Human intestinal wall tissue, from a postmortem sample of upper small intestine, was homogenised and a protein extract prepared as described previously.[6] [4′-^{13}C]Famciclovir (100 m*M*) (4.49 mg in phosphate buffered saline, pH 7.4, 140 μl) was added to intestinal protein extract (6.86 ml). The final dilution of the extract, relative to the original tissue, was

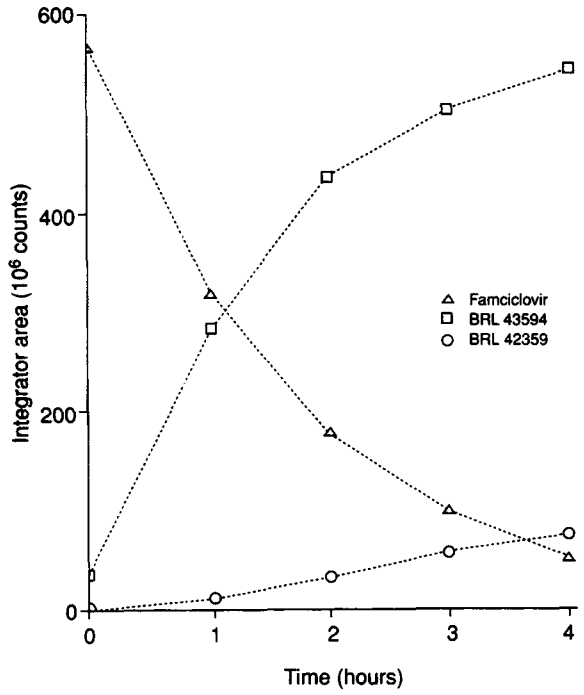

Fig. 3. Incubation of [4'-^{13}C]famciclovir in an extract of human intestinal homogenate. Samples were taken from the incubation at the indicated times and analysed by HPLC, the eluate being monitored at λ = 310 nm for the analysis of the 6-deoxy derivatives of penciclovir.

8.6-fold. At hourly intervals through the incubation, small samples (10 μl) were taken from the incubation mix, diluted with phosphate buffered saline (90 μl), and the protein precipitated by adding 16% trichloroacetic acid (100 μl). After centrifugation, the supernatant (100 μl) was neutralised with saturated $NaHCO_3$ (20 μl) and 400 m*M* $CH_3CO_2NH_4$ pH 6 (120 μl) before immediate HPLC analysis.[6] The deacetylation of famciclovir to monoacetyl-6-deoxy-penciclovir had progressed sufficiently after 4 h incubation (Fig. 3) and so the remaining incubation reaction was terminated at 4.25 h with 1/10 volume 400 m*M* $CH_3CO_2NH_4$ pH 6 and an equal volume of ethanol. After 0.5 h at −20°C followed by centrifugation, the supernatant was separated from the precipitate and dried under vacuum. The residue was redissolved in water (2.1 ml), the solution clarified by centrifugation, and stored at −70°C. Famciclovir and all the metabolites (Fig. 2) were separated by HPLC (injection volumes 1 × 100 μl, 3 × 500 μl, and 1 × ca. 450 μl), the eluate being monitored at λ 254 and 310 nm. To maintain as high a concentration as possible, only the major part of each peak was collected. No other metabolites were detected. The purity of each pooled fraction was checked and the metabolite quantified by HPLC before drying the sample under vacuum.

Analysis of Metabolites

Proton-decoupled ^{13}C NMR spectra of the isolated monoacetyl metabolites were recorded as D_2O solutions on a Bruker AM400 NMR spectrometer operating at a frequency of 100.16 MHz for ^{13}C and using standard software. The ^{13}C NMR chemical shifts were referenced relative to an external standard of dioxan. Peak ratio determination was achieved via peak height analysis and/or instrumental integration.

UV spectroscopy, mass spectrometry, and ^{1}H NMR were used to confirm the identity of the metabolites. UV spectra were recorded using a Kontron Uvikon 810 spectrophotometer. Electron impact and fast atom bombardment mass spectral data were acquired on a VG 7070 or VG ZAB 1F spectrometer. ^{1}H NMR spectroscopy was performed on a Bruker AM400 NMR, referencing ^{1}H chemical shifts to the HOD resonance at 4.8 ppm.

TABLE 1. Yields of isolated metabolites of [4'-^{13}C]famciclovir in an extract of human intestinal wall[a]

Compound		Yield	
BRL number	Structure	mg	nmoles
BRL 42810 (FCV)	Diacetyl-6-deoxy-PCV	0.21	646
BRL 43594	Monoacetyl-6-deoxy-PCV	1.80	6,440
BRL 42359	6-Deoxy-PCV	0.15	625
BRL 39913	Diacetyl-PCV	0.002	6
BRL 42222	Monoacetyl-PCV	0.09	307
BRL 39123 (PCV)	PCV	0.0008	4

[a][4'-^{13}C]Famciclovir (14,000 nmol) was incubated in an extract from human intestinal wall for 4.25 h and the metabolites were extracted and separated as described in the text. The purity of each pooled fraction was checked by HPLC and the amount of metabolite was determined by comparison with synthetic standards.

RESULTS

The progress of the metabolism of famciclovir in the intestinal protein extract was followed by HPLC analysis of a small sample. Only famciclovir and the two 6-deoxy metabolites were assayed as the column eluate was monitored at λ 310 nm. During the 4 h incubation, famciclovir was extensively metabolised, mostly to monoacetyl-6-deoxy-penciclovir (Fig. 2). The yields of famciclovir and its metabolites recovered after purification by HPLC are shown in Table 1. The UV spectra of famciclovir, BRL 43594 and BRL 42359 were typical for 6-deoxyguanines in water (λ_{max} at 242.5 and 304, 242.5 and 304, and 243 and 303 nm, respectively) whereas that of BRL 42222 was typical for guanines (λ_{max} at 252 nm, sh at 275 nm). The fast atom bombardment mass spectra of famciclovir, BRL 43594, BRL 42359, BRL 39913, and BRL 42222, each contained the expected MH^+ adduct ion. For BRL 43594 and BRL 42222, the sodium (MNa^+) and potassium (MK^+) adduct ions also were detected. There was insufficient penciclovir to obtain a satisfactory mass spectrum. The proton NMR spectrum of BRL 43594 confirmed the structure of this metabolite, the NMR spectrum being similar to that obtained previously.[4] The proton NMR spectrum of BRL 42222, although poor due to the small quantity of compound available, did confirm that this metabolite was oxidised on the 6-position of the purine ring, had only one acetyl group and was consistent with the spectrum obtained previously.[10]

The ^{13}C NMR spectrum (Fig. 4a) of isolated monoacetyl-6-deoxy-[4'-^{13}C]penciclovir showed two resonances: a major resonance at 65.5 ppm, assignable to an acetylated hydroxymethyl carbon, and a minor resonance at 62.3 ppm, assignable

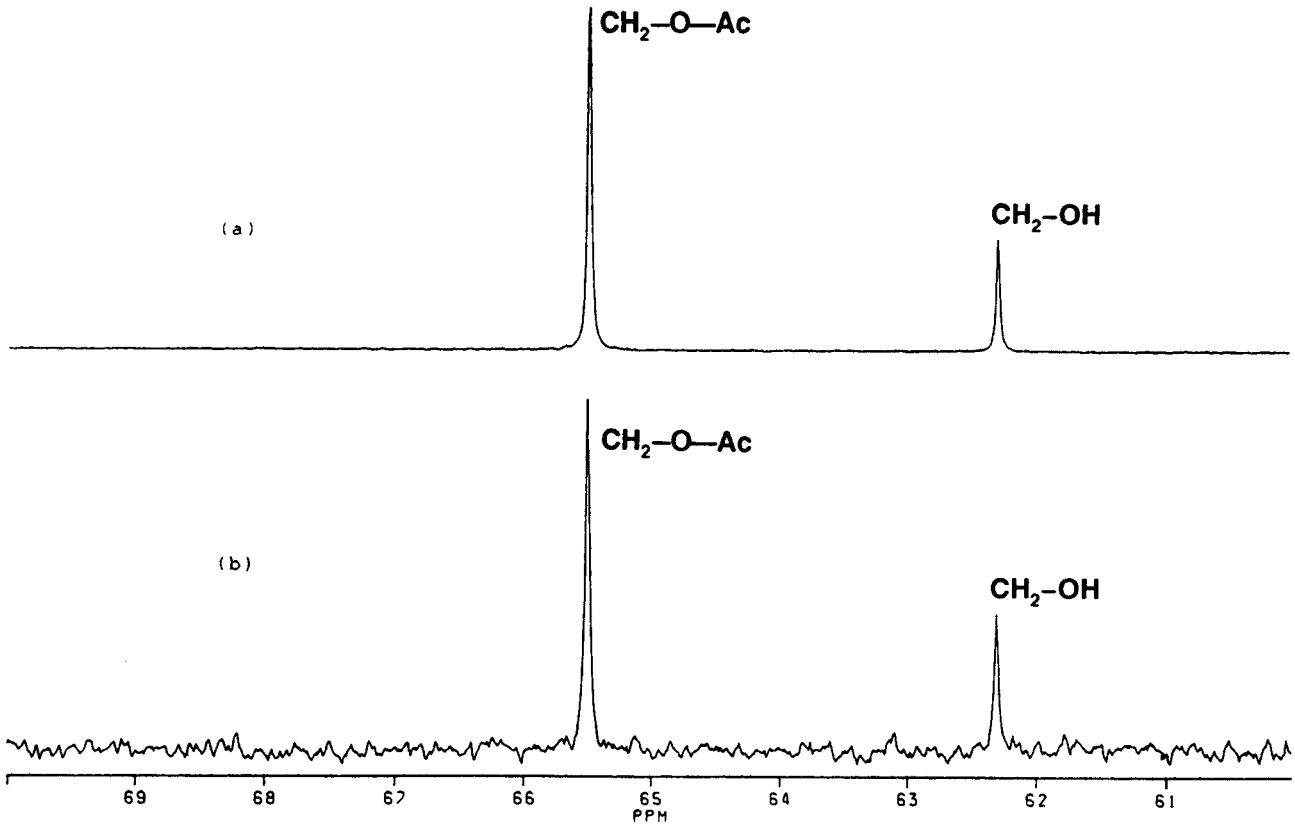

Fig. 4. ^{13}C NMR spectra of chiral metabolites. **(a)** Monoacetyl-6-deoxy-[4′-^{13}C]penciclovir, and **(b)** monoacetyl-[4′-^{13}C]penciclovir isolated when [4′-^{13}C]famciclovir (**1a**) was incubated in an extract of human intestinal wall.

to a hydroxymethyl carbon. Assignment of the ^{13}C NMR resonances was based on known assignments for the unenriched analogues. Instrumental integration of the peak areas yielded a peak ratio about 77:23. The peak ratio determined using the peak height method of analysis was found to be about 76:24.

The ^{13}C NMR spectrum (Fig. 4b) of the isolated monoacetyl-[4′-^{13}C]penciclovir also showed resonances at 65.5 and 62.3 ppm. As in the case of isolated monoacetyl-6-deoxy-[4′-^{13}C]penciclovir, the major signal was that resonating at 65.5 ppm and was assigned to an acetylated hydroxymethyl carbon. Peak height analysis of the ^{13}C NMR resonances yielded a peak ratio about 72:28.

DISCUSSION

The almost exclusive formation of the monoacetylated derivatives of famciclovir (Fig. 3) was similar to that reported previously using the same sample of intestinal tissue.[6] However, as this sample was from a postmortem sample taken 24 h after death, it was of concern that some of the esterase activity may have been lost. Later, when a sample of intestine taken only 2 h after death became available, the conversion of famciclovir in the corresponding protein extract was investigated. Although the levels of esterase activities were about twice those in the previous sample, the preferential formation of the monoacetylated derivatives of famciclovir was similar. In phosphate buffered saline, pH 7.4, famciclovir was stable for the duration of the incubation.

Monoacetyl-6-deoxy-penciclovir and monoacetyl-penciclovir are two of the intermediates formed during conversion of famciclovir to penciclovir. Hydrolysis of one of the acetyl ester groups of famciclovir creates a chiral centre and hence leads to the possible formation of (*R*)- and (*S*)-enantiomers. By using isotopically chiral [4′-^{13}C]famciclovir (**1a**), the enantiomers of the monoacetyl metabolites, produced during incubation with human intestinal wall extract and isolated by HPLC, can be distinguished using ^{13}C NMR spectroscopy. When the acetyl moiety of the isotopically enriched acetoxymethyl group is hydrolysed, the resulting enantiomer will have the ^{13}C label in a hydroxymethyl group and hence will resonate near 62.5 ppm in the ^{13}C NMR spectrum. Conversely, the enantiomer, produced when the acetyl of the unenriched acetoxymethyl group is hydrolysed, will retain the ^{13}C label in an acetoxymethyl group and therefore will give a ^{13}C NMR resonance near 65.5 ppm.

Since the major resonance in the ^{13}C NMR spectra of isolated monoacetyl-6-deoxy-[4′-^{13}C]penciclovir and monoacetyl-[4′-^{13}C]penciclovir was that at 65.5 ppm it can be concluded that the enantiomer retaining the acetyl group on the isotopically enriched hydroxymethyl was formed preferentially. Thus, the esterase(s) in human intestinal wall extract removed preferentially the acetyl ester from the pro-(*S*)-acetoxymethyl group of famciclovir. Furthermore, the ^{13}C NMR-

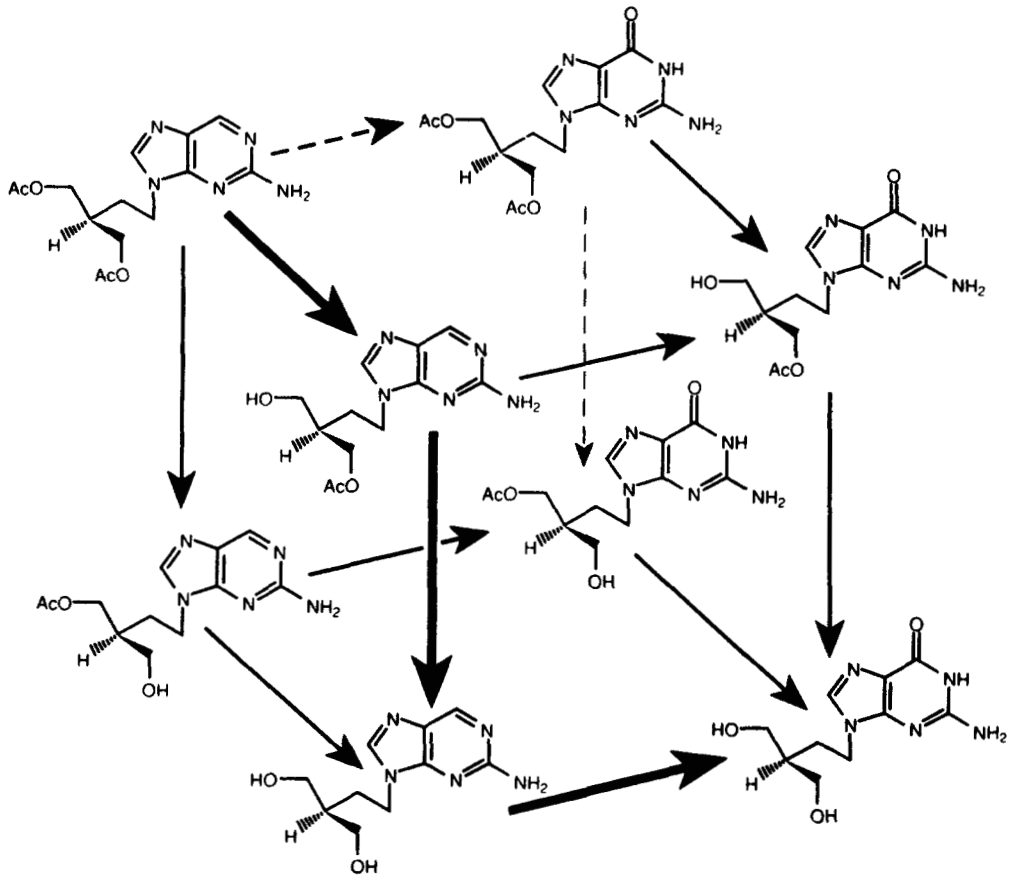

Fig. 5. Conversion of famciclovir to penciclovir. In an extract from human intestinal wall, the major metabolic route (see heavy arrows) is by deacetylation, initially mainly from the pro-(S)-acetoxymethyl group, followed by oxidation of the purine. (Adapted, with permission, from Vere Hodge, 1993, *Antiviral Chemistry & Chemotherapy* 4:67–84, Blackwell Scientific Publications Limited.)

determined peak ratios indicated that the specificity of the esterase(s) was about 77% in the formation of monoacetyl-6-deoxy-penciclovir and about 72% in the formation of monoacetyl-penciclovir.

CONCLUSION

Famciclovir was converted, in a protein extract from human intestinal wall, to penciclovir as indicated in Figure 5. The major route (heavy arrows) was by deacetylation, mainly at the pro-(S)-acetoxymethyl group of famciclovir, followed by hydrolysis of the remaining acetyl group and then oxidation at the 6-position of the purine. However, as all the metabolites (Fig. 5) were detected, there was some metabolism by the other routes, all of which resulted in the conversion of famciclovir to penciclovir.

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