ELSEVIER

Journal of Chromatography A, 889 (2000) 177–184

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Analytical techniques used for monitoring the biodegradation of fluorinated compounds in waste streams from pharmaceutical production

Anthony P. New*, Luisa M. Freitas dos Santos, Giuseppe Lo Biundo, Arnaud Spicq

Environmental Research Laboratory, Analytical Sciences, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park North, Third Avenue, Harlow, Essex, CM17 5AW, UK

Abstract

During the assessment of the environmental impact of new pharmaceutical processes the selection and testing of suitable environmental treatment technologies is carried out. A large component of process waste stream treatment practice is aerobic biotreatment in wastewater treatment plants, as it is cost effective and generally more environmentally friendly than harsher chemical/physical treatments. Pharmaceutical syntheses use a range of halogenated compounds (either as reagents, solvents or intermediates) which pose particular challenges to microbial degradation. This is especially so for some fluorinated compounds due to the resilience to enzymatic cleavage of the C–F bond in some cases. The data presented here were obtained from a case study involving the monitoring of the biodegradation of 4-fluorocinnamic acid by means of a range of chromatographic techniques. These methods were used to monitor not only the disappearance of the compound but also the formation of degradation products in order to confirm mineralisation. In addition mass spectrometry was used to elucidate the metabolic pathway.

Keywords: Process monitoring; Fluorocinnamic acid; Organofluorine compounds

1. Introduction

Waste management is important in the manufacture of pharmaceuticals, in order to minimise both the costs associated with the process and the possible impact that the process may have on the environment.

The preferred method of waste management is the prevention or reduction of waste, for example by cleaner synthesis [1]. This is followed by waste minimisation, which may be managed by computer aided design (see for example [2]). Another method for consideration is recycling. Common methods of recovery of toxic or expensive solvents include liquid–liquid extraction and distillation [3]. Research into alternative methods of solvent recovery involving techniques such as the use of membrane technology [4] are currently ongoing. However, the treatment and disposal of process related waste products is currently inevitable, hence it is necessary to investigate and apply the most suitable treatment and disposal technologies for each process waste. When dealing with aqueous streams, the treatment process usually involves some form of chemical or biological oxidation of the waste stream. Biological oxidation is the preferred method when possible, as it is less expensive and more environmentally friendly than the chemical methods. In order to successfully apply biological oxidation treatment it is necessary to study

*Corresponding author.

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00571-9

the toxicity and biotreatability of each waste stream and often the compounds present in the stream.

Experiments to monitor the biodegradation of individual compounds are usually carried out using chromatographic techniques. It is necessary to monitor both the removal of the test compound and formation of biodegradation products, to infer that biodegradation has occurred, rather than removal due to an artefact of the experiment, for example microbial absorption. Moreover, the use of control experiments are important in this type of study to expose chemical degradation. Further information concerning the products formed may be obtained by the use of an orthogonal technique such as mass spectrometry.

Halogenated compounds are commonly used in the synthesis of pharmaceutical compounds either as reagents, solvents or intermediates, and these pose particular challenges to microbial degradation. In this paper we describe an investigation into the biodegradation of 4-fluorocinnamic acid (4-FCA), a common reagent in the synthesis of pharmaceuticals. The biodegradation experiments employed and the analytical techniques used to monitor the biodegradation and identification of products formed will be described.

2. Experimental

2.1. Biodegradation

Shake flasks (250 ml) containing a solution of mineral salts, activated sludge and 4-FCA (at three concentrations: 50, 100 and 200 mg l^{-1}) were used in duplicate. Activated sludge was obtained from a biological waste water treatment plant at SB in the UK. 4-FCA was supplied by Aldrich, Poole, UK. The composition of the mineral salts solution is shown in Table 1.

The flasks were placed in an Innova 4000 incubated orbital shaker (New Brunswick Scientific Co., New Brunswick, USA). This was set to a temperature of 25°C and a shake speed of 150 rpm. The flask labels and compositions are shown in Table 2. Samples were taken from the flasks at regular intervals over a 149 h period. The solutions were filtered using 0.2 μm Minisart filters, (Sartorius, Göttingen, Germany), in order to remove the bacteria and debris from the samples and hence stop the reaction prior to analysis. Samples were refrigerated between analyses at 0–4°C in polyethylene plastic vials.

Table 1
Composition of mineral salts solution

Compound	Formula	Concentration (mg l^{-1})
Magnesium sulphate	$MgSO_4$	22.5
Potassium dihydrogenphosphate	KH_2PO_4	85.0
Potassium hydrogenphosphate	K_2HPO_2	220
Sodium hydrogenphosphate	Na_2HPO_4	330
Ammonium chloride	NH_4Cl	5.0
Iron chloride	$FeCl_3 \cdot 6H_2O$	0.25
Calcium chloride	$CaCl_2$	27.5
Calcium chloride	$CaCl_2 \cdot 2H_2O$	36.4

2.2. High-performance liquid chromatography (HPLC)

HPLC was carried out using a Hewlett-Packard HP 1100 (Hewlett-Packard, Palo Alto, CA, USA) HPLC system equipped with a NovaPak C_{18} column 150 mm×150 mm×3.9, 5 μm particle size (Waters, Watford, UK). An injection volume of 20 μl was used. The flow rate was set to 0.8 ml min^{-1}, the UV detector wavelength was 230 nm and the column oven temperature was set to 40°C. The eluent consisted of (0.02 *M* ammonium acetate adjusted to pH 4.5 with glacial acetic acid)–methanol (70:30, v/v). The run was isocratic for 10 min.

Table 2
Experiment to test biodegradation of 4-FCA: flask composition

Flask number	Initial 4-FCA concentration (mg l^{-1})	Experiment
1	200	Active
2	200	Active
3	100	Active
4	100	Active
5	50	Active
6	50	Active
7	100	Control
8	100	Control

2.3. Ion chromatography

Ion chromatographic analysis was carried out using a Dionex DX 500 ion chromatograph (Dionex, Sunnyvale, CA, USA), equipped with an AS11 analytical column (250×4 mm) and an AG11 guard column (50×4 mm), also supplied by Dionex. An injection volume of 10 μl was used. The column temperature was set to 30°C and flow rate 1.0 ml min^{-1}. Detection was by suppressed conductivity. An hydroxide gradient was produced electrically using a Dionex EG40 eluent generator (supplied by Dionex). This was $t=0$; 5 m*M* hydroxide, $t=5$ min; 5 m*M* hydroxide, $t=7$ min; 45 m*M* hydroxide, $t=10$ min; 45 m*M* hydroxide, $t=12$ min; 5 m*M* hydroxide, $t=15$ min; 5 m*M* hydroxide.

2.4. Gas chromatography–mass spectrometry (GC–MS)

GC–MS was carried out using a Hewlett-Packard MSD 6870, equipped with an SGE BP1 25 m×0.22 mm I.D. column, 0.25 μm film thickness. The temperature program was as follows: 50°C for 2 min, then 10°C min^{-1} to 150°C, then 30°C min^{-1} to 210°C. A post run time of 1.0 min gave a total run time of 15 min. The inlet mode was pulsed split, with a pulse pressure of 5.0 p.s.i. for 1.0 min and a split ratio of 20:1 and a split flow of 23.1 ml min^{-1} (1 p.s.i.=6894.76 Pa). Helium was used as a carrier gas with a flow rate of 1.2 ml min^{-1} in constant flow mode. The nominal initial pressure was 13.64 p.s.i. and the average velocity was 43 cm s^{-1}. The injection volume was 1 μl.

The MS transfer line temperature was set to 280°C. The mass spectrometer source temperature was set to 230°C and the quadrupole temperature to 150°C. The ionisation mode was electron ionisation (EI) and the quadrupole was set to scan from m/z 25–550. The electron multiplier voltage was set to 1188 volts.

2.5. Liquid chromatography–mass spectrometry (LC–MS)

LC–MS was carried out using a Hewlett-Packard HP 1090 (Hewlett-Packard, Palo Alto, CA, USA) linked to a Micromass Q-TOF (Micromass, Manchester, UK) mass spectrometer. The HPLC method

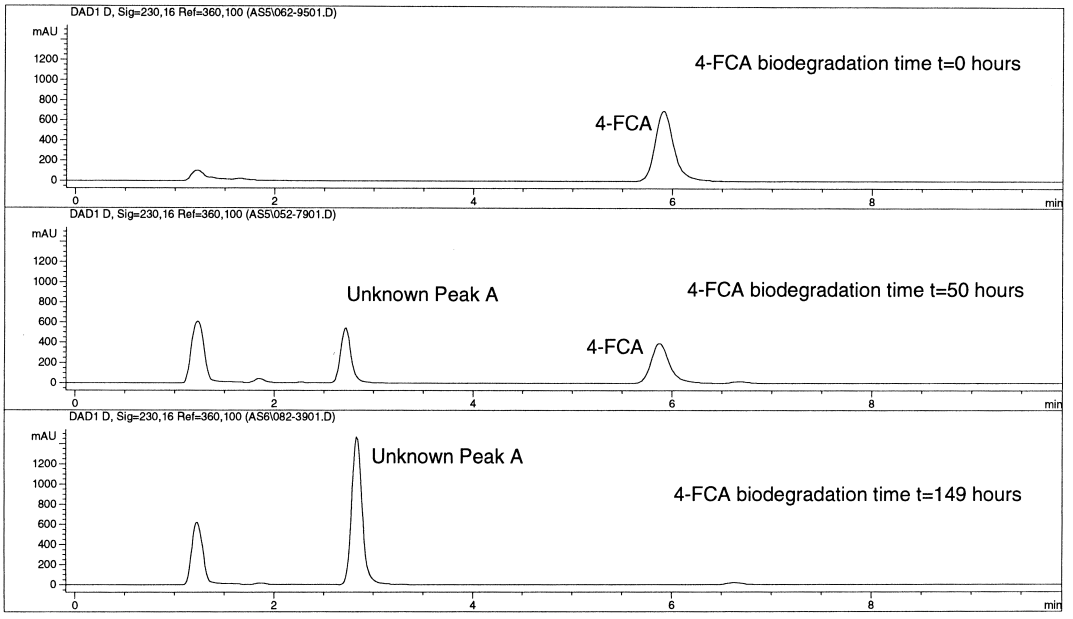

Fig. 1. HPLC analysis of solutions taken from flask fed with 200 mg l^{-1} fluorocinnamic acid during biodegradation experiments (flask 2).

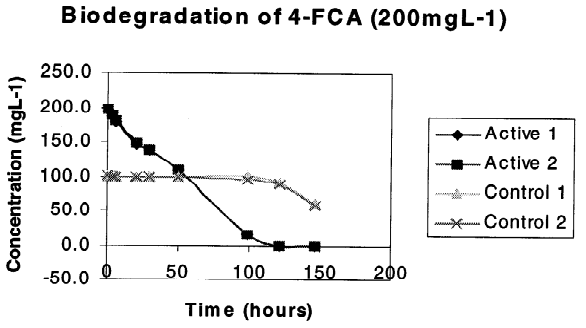

Fig. 2. Graph to show removal of 4-FCA during biodegradation reaction.

was as above except that a 50 μl injection volume was used.

The mass spectrometer was used in negative ionisation mode with a cone voltage of 20 V, source block temperature of 100°C, desolvation temperature of 300°C, mass range of 60–1000 and a scan cycle time of 1 s.

3. Results and discussion

3.1. High-performance liquid chromatography

The amount of 4-FCA in each of the samples taken from the active and control flasks over the time period of the experiment was estimated using HPLC. Fig. 1 shows the chromatography obtained for the flasks initially containing 200 mg l^{-1} of 4-FCA.

The removal of 4-FCA from the solutions supplied with 200 mg l^{-1} is shown in Fig. 2. It is noted that after 50 h the amount of 4-FCA was reduced to 100 mg l^{-1} and after 100 h was not detected in the active flask. The graph shown in Fig. 2 also shows that the amount of 4-FCA in the control flask remains constant inferring that there is no chemical degradation of this compound. However, it is noted that, the last two points at 122 h and 148 h show some removal of 4-FCA from the control. This was probably due to cross contamination of the control flasks since sterile conditions were not used. Since it can be clearly seen in Fig. 2, that all of the 4-FCA was removed from the active flasks after 100 h and the control flasks were unaffected up to this point, it was assumed that the experiment to investigate biodegradability of the 4-FCA was unaffected by this observation.

The half lives for removal from these solutions supplied with 200 mg l^{-1}, 100 mg l^{-1} and 50 mg l^{-1} were calculated as 53.3 h, 20.4 h and 5.4 h, respectively.

3.2. Ion chromatography

In previous experiments halide production has been shown to occur on biodegradation of a number of halogenated compounds (see for example Ref.

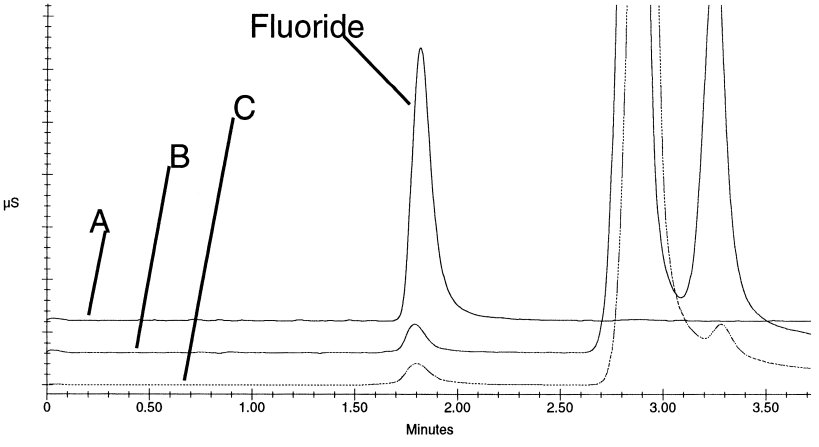

Fig. 3. Portion of ion chromatogram from 0 to 3.60 min. Trace A=10 ppm standard fluoride, trace B=sample taken from flask 2 after 149 h, Sample C sample taken from flask 2 at time t=0 h (initial 4-FCA concentration 200 mg l^{-1}.

[5]). Hence, ion chromatography was used to investigate the possible production of fluoride and attempt to correlate the production of fluoride with the removal of 4-FCA from each flask.

Fig. 3 shows an example of the type of chromatography obtained and illustrates that there is only a small increase in fluoride over the time period of the experiment. Table 3 shows the amount of fluoride measured in each of the samples.

Table 3 shows the % recovery of fluoride for each concentration supplied. The low recoveries shown in Table 3 correlated to the HPLC data in each case suggests the formation of fluorinated metabolites. There appears to be a slightly higher recovery for the lower concentration (50 mg l^{-1}) supplied. As the half life for removal of 50 mg l^{-1} is low (5.4 h) it is possible that the fluorinated metabolite(s) formed initially were being removed as the biodegradation proceeded towards the hydrolysis of the fluoride and complete mineralisation. Since the concentrations of fluoride formed in each case are quite small, this would require further investigation, involving the use of higher initial concentrations of 4-FCA or using a longer time period for the experiment.

3.3. Liquid-chromatography–mass spectrometry

In order to attempt to identify the degradation product observed in the HPLC analysis, LC–MS was carried out. Fig. 4 shows the LC–MS data acquired. There is good agreement between the UV and total ion current (TIC) traces. A mass spectrum was only obtainable on peak A, the major degradation product observed in the HPLC chromatograms. This peak gave the spectrum shown in Fig. 5. The accurate mass measurement of 139.0190 agrees with the theoretical value (139.0195) to within 5 ppm. This is consistent the observation made by GC–MS. The remaining ions in the spectrum are due to adducts with eluent formed in the mass spectrometer.

Table 3
Fluoride concentration for each sample during the biodegradation of 4-FCA

Initial 4 FCA concentration (mg l^{-1})	Theoretical fluoride yield[a] (mg l^{-1})	Average fluoride concentration measured after 149 h (mg l^{-1})	Recovery (%)
200	22.9	1.2	5.3
100	11.5	0.6	5.2
50	5.6	0.8	14.2

[a] Assuming total mineralisation.

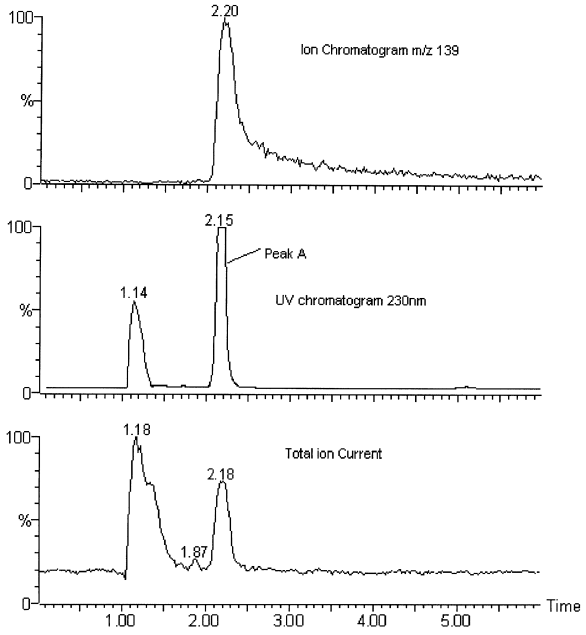

Fig. 4. LC–MS analysis of the sample taken from the flask supplied with 200 mg l^{-1} 4-FCA (Flask 2) after 149 h. Time scale in min.

3.4. Gas chromatography–mass spectrometry

A GC–MS investigation was carried out to attempt to identify metabolites of the 4-FCA. The TIC for the GC–MS chromatogram is shown in Fig. 6.

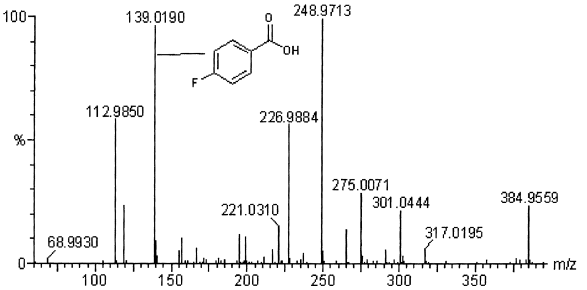

Fig. 5. LC–MS spectrum of peak A from the chromatogram shown in Fig. 4.

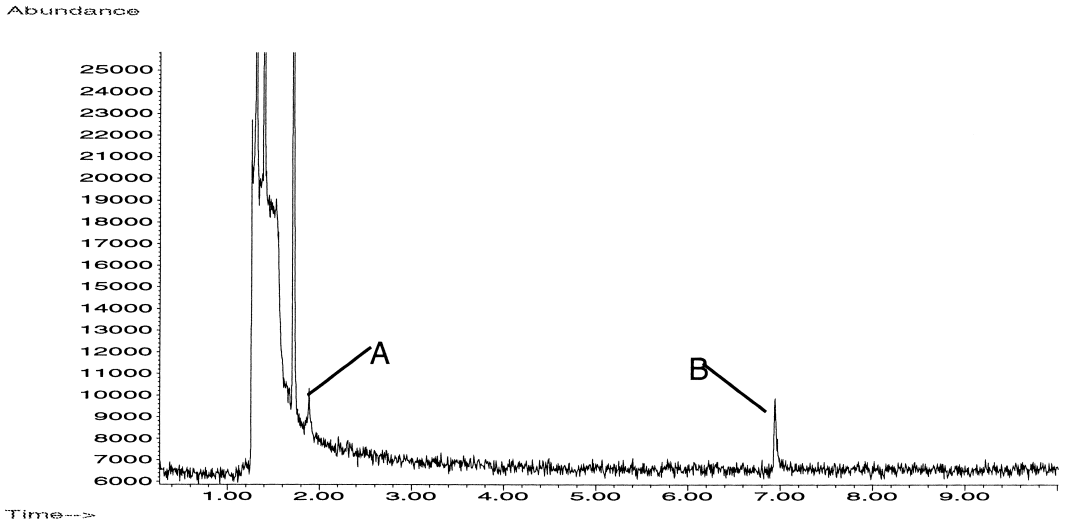

Fig. 6. GC–MS trace for sample taken at time 149 h, initial feed 200 mg l^{-1} (Flask 2). Time scale in min.

Two of the peaks in the chromatogram are aromatic fluorine containing compounds identified as 4-fluorobenzoic acid and 4-fluoroacetophenone by a library search. The mass spectra for these compounds are shown in Figs. 7 and 8.

The mass spectra shown in Figs. 7 and 8 were identified by comparison with the NIST98 library within the HP MSD software and peak A was found to be 4-fluorobenzoic acid which is consistent with the LC–MS data. Peak B was found to be 4-fluoroacetophenone. Using this information and with reference to the literature [6,7] a pathway for biodegradation of 4-FCA is proposed and shown in Fig. 9.

The formation of benzoic acids from cinnamic acids has previously been shown, for example the conversion of ferrulic to vanillic acid was achieved. However, the degradation of vanillic acid was not possible under the conditions used [8]. The pathway proposed in Fig. 9 shows that the major product of the metabolism is 4-fluorobenzoic acid. The route suggests that 4-FCA is decarboxylated, then oxidised to the diol which loses water to give an epoxide.

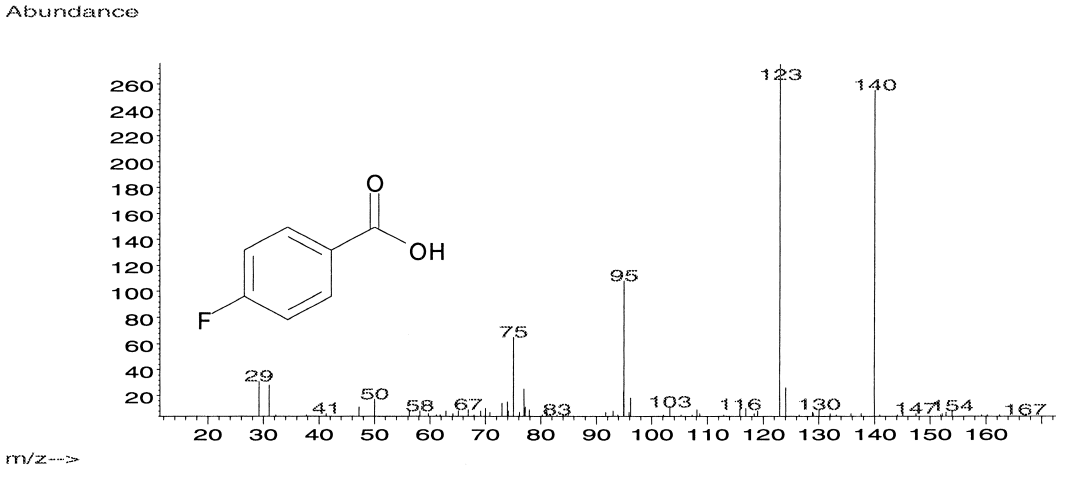

Fig. 7. Mass spectrum for peak A of Fig. 6.

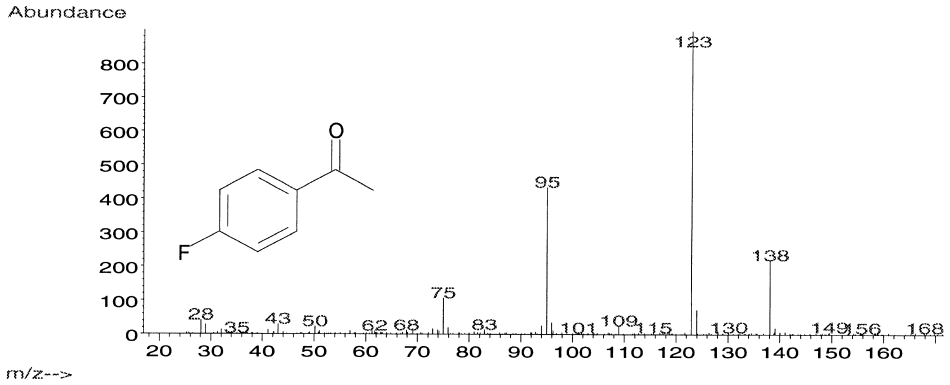

Fig. 8. Mass spectrum for peak B of Fig. 6.

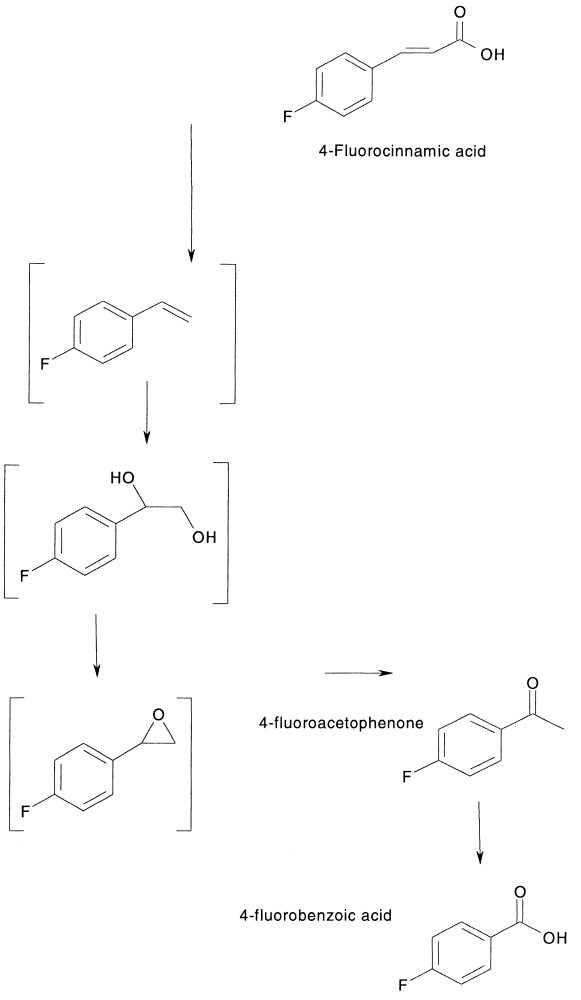

Fig. 9. Proposed pathway of biodegradation for 4-fluorocinnamic acid. Components in brackets indicate suggested pathway.

However none of these intermediates were observed and further work would be needed to confirm this. It is further proposed that the epoxide would rearrange to give 4-fluoroacetophenone which would be further oxidised to the major product 4-fluorobenzoic acid. The overall result of this proposed pathway is that the molecule is reduced from nine carbon atoms to seven.

We have carried out similar experiments to investigate the biodegradation of 4-fluorobenzoyl chloride (hydrolysed to 4-fluorobenzoic acid in aqueous solutions) and have shown that it is biodegradable with the formation of stoichiometric amounts of fluoride. These data are summarised in Fig. 10 and observation is consistent with previous work [6,7].

4. Conclusions

A number of analytical techniques were used to ascertain that 4-FCA is biodegradable. The major metabolite was identified as 4-fluorobenzoic acid which is also biodegradable. From this work it may be predicted that, on supplying streams containing 4-FCA to a biological waste water treatment plant, complete mineralisation would be achieved.

Acknowledgements

We thank Jean-Claude Wolff for acquiring and interpreting the LC–MS data.

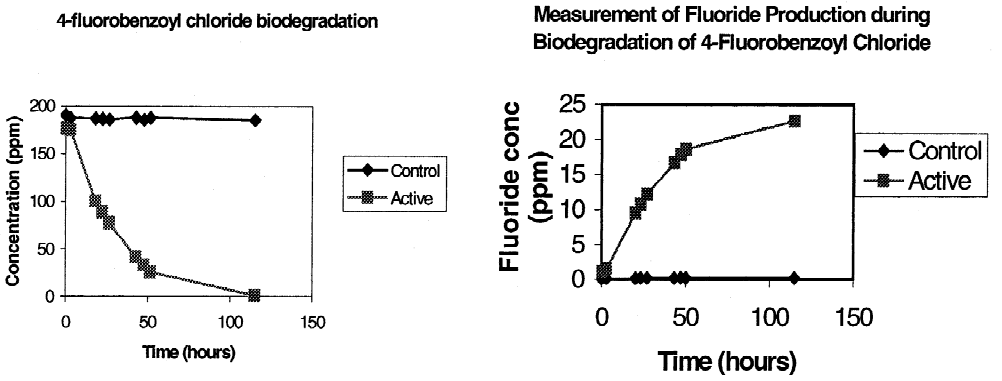

Fig. 10. Summary of biodegradation experiments for 4-fluorobenzoyl chloride.

References

[1] J. Clark, Chem. Brit. 34 (10) (1998).
[2] Batch Plus Modelling, Aspentech Inc., Cambridge, MA.
[3] A. Kirk-Othmer, Encyclopedia of Separation Technology, Wiley–Interscience, New York, 1997.
[4] W. Ho, K. Sirkar (Eds.), Membrane Handbook, Chapman & Hall, London, 1992.
[5] L.M. Freitas dos Santos, D. Lamarca, M. Gilges, A.P. New, Trans. IChemE 77 (Part B) (1999).
[6] R.H. Oltmanns, R. Mueller, M.K. Otto, F. Lingens, Appl. Environ. Microbiol. 55 (1989) 2499.
[7] C.A. Fewson, S.I.T. Kennedy, A. Livingstone, J. Biochem. 109 (1968) 6P.
[8] M. Ruzzi, P. Barghini, F. Montebove, A. Sciesser, Ponente Ann. Microbiol. Enzymol. 47 (1997) 87.