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Degradation study of the investigational anticancer drug clanfenur

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Abstract

Clanfenur belongs to a new group of substituted benzoylphenylureas. The drug shows both in vitro and in vivo antitumour activity. To assess its chemical stability, a study was carried out in which the effect of pH, temperature, ionic strength and buffer concentration on the reaction rate constant k_{obs} were examined. A stability-indicating reversed-phase high performance liquid chromatography (RP-HPLC) system was used. The pH-log k_{obs} degradation profile, obtained at 70°C, shows that clanfenur has its maximum stability in the pH region 4–5. At pH 7, half-lives were calculated by extrapolation of the Arrhenius plot; at 4°C the half-life was calculated to be 141 years and at 25°C 9.5 years. The activation energy was calculated to be 114 kJ/mol. In acidic, neutral, and alkaline media, the ionic strength has no effect on the degradation. The buffer concentration of citrate, phosphate, borate, and carbonate did not affect the value of k_{obs}. An RP-HPLC chromatogram of degraded clanfenur shows the presence of four degradation products, three of which were identified by LC-ESI-MS as *p*-chloroaniline, *p*-chlorophenylurea and 2-fluoro-6-dimethylaminobenzamide. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Clanfenur; Cytostatic; Stability; Degradation products; LC-ESI-MS

1. Introduction

The investigational anticancer drug clanfenur (*N* - {[(4′ - chlorophenyl)amino]carbonyl} 2″ - (dimethylamino) - 6″ - fluorobenzamide (DU 113901), Fig. 1(1)) is a substituted benzoylphenylurea and an analogue of the pesticide diflubenzuron (Fig. 1(2)). Diflubenzuron was the first benzoylphenylurea reported as a growth regulator; the compound itself and analogues such as clanfenur might, therefore, affect tumour growth. Several studies have dealt with this aspect: diflubenzuron showed antitumour activity in B 16

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melanoma and in skin tumours (Jenkins et al., 1984, 1993).

Also for clanfenur, in vitro as well as in vivo antitumour activity was demonstrated (Jonkman-de Vries et al., 1997). Hofs et al. (1988) selected clanfenur for development in clinical studies because of its in vitro antitumour activity against human tumour cells, its lack of mutagenicity in the Ames test, and the absence of toxicity in rodents and dogs. In addition, evaluation of the mode of action of clanfenur showed a cell cycle-dependent action without cell phase specificity. Evaluation of the antitumour activity was carried out in a rat liver metastasis model. Clanfenur cleared all metastases in this model and no toxicity was observed (Hofs et al., 1988).

The cytostatic activity of several structurally modified benzoylphenylureas was examined in vitro in order to establish a structure–activity relationship. In B16 melanoma cells, the introduction of a dimethylamino group at the 2-position of the benzoyl ring, as in clanfenur, led to a distinctly higher activity compared to the parent compound diflubenzuron (Hofs and McVie, 1991).

Several analytical methods have been reported for the determination of benzoylphenylureas in various matrices, in particular for the analysis of the pesticide diflubenzuron in environmental and agricultural samples: high performance liquid chromatography with ultraviolet detection (HPLC-UV) (Prima et al., 1978; Sundaram and Nott, 1989), gas chromatography-mass spectrometry (GC-MS) (Wimmer et al., 1991), and HPLC-MS (Barnes et al., 1995). A selective RP-HPLC method for the determination of clanfenur and its metabolites in rat and human plasma was developed by Noteborn et al. (1994). This method is well-suited for pharmacokinetic studies in preclinical and clinical trials.

Clanfenur is poorly soluble in water (0.4 μg/ml) and saline (0.25 μg/ml). This raises problems to reach target plasma levels needed for optimal therapeutic efficacy. Jonkman-de Vries et al. (1997), therefore, explored drug formulations with which the required plasma concentrations could be reached. To determine storage and administration conditions for the optimum formulation, clanfenur in Cremophor EL®/ethanol (1:1, w/v), they carried out an accelerated stability test. From their study a long-term stability of 3.5 years at 4°C and 4 months at 25°C was calculated. They found that a chromatogram of degraded clanfenur shows four degradation products, of which

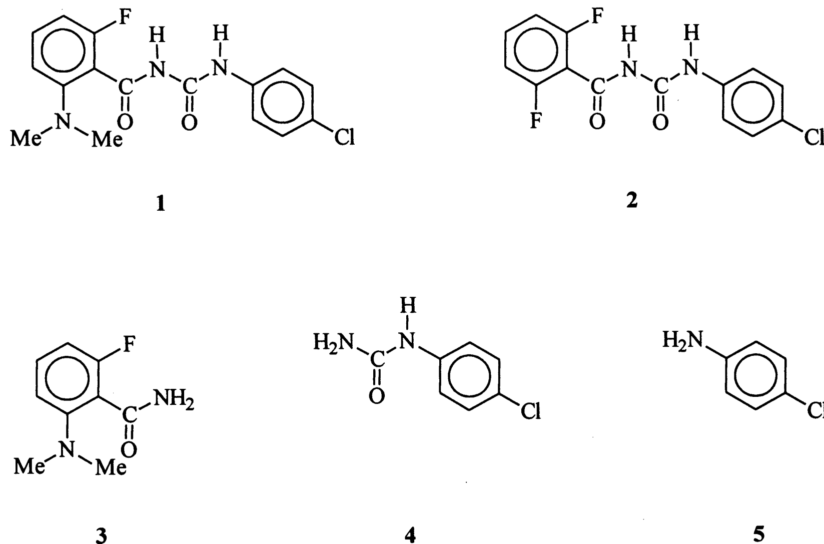

Fig. 1. Structure of clanfenur (1), diflubenzuron (2), and the degradation products 2-dimethylamino-6-fluorbenzamide (3), *p*-chlorophenylurea (4), and *p*-chloroaniline (5).

p-chloroaniline (Fig. 1(5)) was identified on the basis of its capacity factor. The structures of the other products were not determined. However, the identity of the degradation products is important in predicting possible side-effects and toxicity. For example, *p*-chloroaniline, which was also found as a degradation product of diflubenzuron, was shown to be a borderline mutagen in the Ames test (Seuferer et al., 1979).

The present paper describes a systematic degradation study of clanfenur in aqueous solution, monitoring the effect of pH, temperature, ionic strength and buffer concentration. The structure of three of the four degradation products was established by LC-Electro Spray Ionisation-MS (LC-ESI-MS).

2. Materials and methods

2.1. Chemicals

For aqueous solutions MilliQ water was used. Suppliers and data for all chemicals used are summarised in Table 1.

Table 1
Summary of chemicals used

Chemical	Manufacturer/supplier
Acetic acid 100%	Merck (Darmstadt, Germany)
Ammonium acetate p.a.	Merck (Darmstadt, Germany)
Citric acid monohydrate p.a.	Merck (Darmstadt, Germany)
Clanfenur	Solvay Duphar (Weesp, The Netherlands)
p-Chloroaniline	Merck (Darmstadt, Germany)
Methanol HPLC	Biosolve Ltd. (Barneveld, The Netherlands)
Potassium hydrogen carbonate p.a.	Merck (Darmstadt, Germany)
Perchloric acid 70–72 % p.a.	Merck (Darmstadt, Germany)
Sodium acetate p.a.	Merck (Darmstadt, Germany)
Sodium chloride p.a.	Merck (Darmstadt, Germany)
Sodium bicarbonate c.p.	Acros Organics (New Jersey, USA)
Sodium dihydrogen phosphate monohydrate p.a.	Merck (Darmstadt, Germany)
Sodium heptane-1-sulfonate 98%	Aldrich (Steinheim, Germany)
di-Sodium hydrogen phosphate dihydrate	Merck (Darmstadt, Germany)
Sodium hydroxide p.a.	Janssen Chimica (Geel, Belgium)
di-Sodium tetraborate cryst. p.a.	Merck (Darmstadt, Germany)

2.2. HPLC

Two different HPLC systems were used. HPLC system I was used for calibration, method validation, determination of the standard deviation in k_{obs}, pH-log k_{obs} profile, Arrhenius plot, and determination of the effect of the ionic strength and buffer concentration. HPLC system II was used for the characterisation of the degradation products by LC-MS.

2.2.1. HPLC system I

The solvent delivery system was a model M-6000 pump (Waters Associates, Milford, MA, USA). A U6K injector (Waters Associates) with an injection volume of 20 μl and a 50942 LiChroCART 125-4 LiChrospher® 100 RP-8 analytical column (particle size 5 μm, 125 × 4 mm) (Merck, Darmstadt, Germany) were used. The flow rate was 0.5 ml/min and the column was at room temperature. Detection was carried out at a wavelength of 254 nm with a model 440 detector (Waters Associates). The clanfenur peak area was integrated with an SP 4290 Integrator (Spectra-Physics, Eindhoven, The Netherlands). The mobile phase was methanol/10 mM citric acid buffer pH 2.8 (700:300, v/v), containing 10 mM heptane-l-sulfonic acid sodium salt as the ion-pairing agent.

2.2.2. HPLC system II

This system consisted of an LC-10AD pump (Shimadzu Benelux, 's-Hertogenbosch, The Netherlands), a U6K injector (Waters Associates), with an injection volume of 100 μ1, and a 16862 LiChroCART 125-2 Supersphere 100 RP-18 ana-

lytical column (particle size 4 μm, 125 × 2 mm) (Merck). The flow rate was 100 μl/min and the column was at room temperature. UV detection was carried out at a wavelength of 254 nm with an Applied Biosystems 785A programmable absorbance detector (Separations, Hendrik-Ido-Ambacht, The Netherlands), equipped with a 75-μm capillary cell. The mobile phase was methanol/2 mM ammonium acetate, pH 6.3 (550:450, v/v).

2.3. Method validation

Peak purity in HPLC system I was assessed with a 1040 A photodiode-array detector (Hewlett-Packard, Palo Alto, PA, USA).

Calibration curves of standard clanfenur solutions were measured with HPLC system I in the range from 5.0×10^{-4} to 1.0×10^{-7} M. Stock solutions of clanfenur were made in methanol. The final solutions for injection into the HPLC system contained the same amount of methanol as the mobile phase. All clanfenur standard solutions were prepared and measured in duplicate. The limit of detection, defined as the signal-to-noise ratio of 3, was calculated from the calibration curve in the range from 1.0×10^{-6} to 1.0×10^{-7} M by measurement of the peak height. The between-day and within-day precision of injection was calculated from duplicate injections of duplicate solutions.

2.4. UV spectra

UV spectra of clanfenur were run at a pH range 0–13 in steps of 0.5, from 340 to 220 nm, on a Hitachi model 100-600 Spectrophotometer (Merck). Water containing 10% methanol was used as the solvent.

2.5. Degradation conditions

Kinetics of degradation were studied by measuring the decrease of the clanfenur concentration (peak area) in the degradation samples as a function of time. For determination of the standard deviation in k_{obs}, pH-log k_{obs} profile, Arrhenius plot, and determination of the effect of the ionic strength and buffer concentration, samples were prepared as follows: in 1-ml ampoules, 900 μl of buffer of appropriate pH, buffer concentration, and ionic strength were added to 100 μl of a 2×10^{-4} M clanfenur solution in methanol, resulting in an initial clanfenur concentration of 2×10^{-5} M. Degradation was monitored over at least three half-lives. Samples were removed from the water bath at appropriate time intervals and diluted 1:9 with mobile phase. The diluted samples were kept at 4°C until HPLC analysis.

For identification of the degradation products by LC-MS, acidic (pH l, perchloric acid), neutral (pH 7, 10 mM phosphate), and alkaline (pH 13, sodium hydroxide) degradation samples (all μ(NaCl) = 0.3) were prepared. To 200 μl of a 1×10^{-3} M clanfenur solution in methanol, 800 μl of buffer was added in 1-ml ampoules, resulting in an initial clanfenur concentration of 2×10^{-4} M. These samples were incubated at 80°C for at least three half-lives. After removal of the samples from the water bath, 400 μl of degraded sample was diluted with 600 μl methanol/2 mM ammonium acetate (500:500, v/v). The diluted samples were kept at 4°C until LC-MS analysis.

2.6. Standard deviation in k_{obs}

The standard deviation in k_{obs} was determined at pH 7 (10 mM phosphate μ(NaCl) = 0.3); degradation of the samples was carried out at 80°C.

2.7. pH-log k_{obs} profile

The degradation of clanfenur was measured in the region from pH/H_0 0 to 13 by measuring the peak area of clanfenur as a function of time. Degradation was carried out in buffered solutions (pH range 1–2 perchloric acid; pH 3 10 mM citric acid; pH range 4–5 10 mM acetate; pH range 6.5–7 10 mM phosphate; pH range 8–9.5 10 mM borate; pH range 10–11 10 mM carbonate; pH range 11.5–12 10 mM phosphate; pH range 12.5–13 sodium hydroxide); the ionic strength μ(NaCl) was 0.3, and the temperature 70°C. The solution with pH/H_0 value 0 was prepared according to Hammett and Deyrup (1932).

2.8. Arrhenius plot

The degradation of clanfenur was monitored in a buffer solution at pH 7 (buffer concentration 10 mM phosphate, ionic strength $\mu(NaCl) = 0.3$). Degradation was carried out at 60, 70, 80, 90, and 100°C.

2.9. Effect of ionic strength

The effect of the ionic strength on the degradation of clanfenur was determined in acidic, neutral, and alkaline media. Samples were prepared at pH 1 (perchloric acid), pH 7 (10 mM phosphate), and pH 13 (sodium hydroxide). The ionic strength varied from 0.1 to 0.9.

2.10. Effect of buffer concentration

The effect of the buffer concentration was examined with phosphate (pH 7) and carbonate (pH 10.5) at concentrations of 0.01 and 1.0 M, and with citrate (pH 3) and borate (pH 9.5) at concentrations of 0.01 and 0.10 M. Degradation was carried out at 80°C, and $\mu(NaCl) = 0.3$.

2.11. Characterisation of degradation products by photodiode-array detection

UV spectra of all degradation products in acidic, neutral and alkaline media were run with a photodiode-array detector, with HPLC system I.

2.12. Characterisation of degradation products by LC-MS

Mass spectra were obtained with a VG Platform II Benchtop mass spectrometer with Masslynx 2.0 data software (both Micromass, Altrichem, UK). An electrospray interface was used to ionise the molecules. The nebulising gas had a flow of 17 l/h, the drying gas 380 l/h. The applied voltage on the capillary was 3.6 kV. Fragmentation of clanfenur was studied in both positive and negative ion mode with loop injections, by varying the cone voltage. For LC-MS, the cone voltage was set at 20 V and 70 V for the positive and negative ion mode, respectively. The mass was calibrated from 10 to 1000 Da, in the positive ion mode with a sodium iodide/triethylamine solution and in the negative ion mode with a sodium iodide solution. LC-MS was carried out with HPLC system II. The column effluent was diverted from the MS for the first 5 min of the chromatogram, to avoid contamination of the MS with unretained components such as salts.

3. Results and discussion

3.1. Method validation

UV spectra run with a photodiode-array detector did not change during elution of the clanfenur peak in a partly degraded sample. Likely degradation products do not coelute with the clanfenur peak; moreover, their UV spectra differ from that of the parent compound.

In HPLC system I, the capacity factor of clanfenur was 4.27 (SD = 0.080, $n = 10$), the variability being caused by the ion-pairing agent. Calibration curves of standard solutions of clanfenur measured with HPLC system I were linear over the range from 5.0×10^{-4} to 1.0×10^{-7} M. The limit of detection, defined as the signal-to-noise ratio of 3, was 0.8 ng. The between-day and within-day precision of injections varied 1.6% and 1.3%, respectively, after duplicate injections.

3.2. Standard deviation in k_{obs}

The mean value of k_{obs} at pH 7 was $(5.0 \pm 0.5) \times 10^{-6}$/s (n = 5).

3.3. Order of degradation reaction

The relation between ln(peak area of clanfenur) and time is linear when measured over at least three half-lives, indicating a (pseudo-)first-order degradation mechanism.

3.4. pH-log k_{obs} profile

Clanfenur possesses one basic function (the dimethylamino group), and two weak acid functions (the benzoylurea group). Therefore, it par-

ticipates in three protolytic equilibria and its pH-k_{obs} profile is described by the following equation (van der Houwen et al., 1988)

$$k_{obs} = \frac{M_0 \cdot [H^+] + M_1 + \frac{M_2}{[H^+]} + \frac{M_3}{[H^+]^2} + \frac{M_4}{[H^+]^3} + \frac{M_5}{[H^+]^4}}{1 + \frac{K_1}{[H^+]} + \frac{K_1 \cdot K_2}{[H^+]^2} + \frac{K_1 \cdot K_2 \cdot K_3}{[H^+]^3}} \quad (1)$$

in which M_0 to M_5 are the macro reaction constants described by Eqs. (2)–(7).

$$M_0 = k_0^H. \quad (2)$$

$$M_1 = k_1^H \cdot K_1 + k_0^S. \quad (3)$$

$$M_2 = k_2^H \cdot K_1 \cdot K_2 + k_1^S \cdot K_1 + k_0^{OH} \cdot K_w. \quad (4)$$

$$M_3 = k_3^H \cdot K_1 \cdot K_2 \cdot K_3 + k_2^S \cdot K_1 \cdot K_2 + k_1^{OH} \cdot K_w \cdot K_1 \quad (5)$$

$$M_4 = k_3^S \cdot K_1 \cdot K_2 \cdot K_3 + k_2^{OH} \cdot K_w \cdot K_1 \cdot K_2. \quad (6)$$

$$M_5 = k_3^{OH} \cdot K_w \cdot K_1 \cdot K_2 \cdot K_3. \quad (7)$$

In these equations the individual reaction constants k are specified with subscripts for the reacting species and with superscripts indicating the type of catalysis. The reacting species are numbered from 0 (fully protonated) to 3 (fully deprotonated). Specific acid catalysis is indicated with the superscript 'H', solvent catalysis with 'S' and specific base catalysis with 'OH'.

With Eq. (1), the macro reaction constants and the pK_a values were calculated from the observed degradation rates by non-linear regression analysis. The results are given in Table 2, and the pH-k_{obs} profile in Fig. 2. This three-pK_a model gives an excellent fit, whereas the two- and one-pK_a model did not. Eqs. (3)–(7) show the potential contributions of kinetically indistinguishable reactions. Maximum values for the individual reactions can be calculated by attributing the macro reaction constant to a single individual reaction, and neglecting the contribution of the other reaction(s). By this procedure the log k values of Table 2 were obtained. Comparison of M_0 and M_1 suggests that M_1 results mainly from acid-catalysed degradation. The reaction constant k_1 describes the reaction of an uncharged species, whereas k_0 refers to a positively charged particle. The latter reaction constant is assumed to be orders of magnitude smaller than the former one. By the same reasoning M_2 is also attributed to acid-catalysed degradation. By analogous reasoning M_3 and M_4 mainly result from base-catalysed degradation.

Both acid- and base-catalysed degradation result in the same degradation products. Therefore, and because part of the degradation products eluted in the void volume, the change in the concentration of observed degradation products as a function of time does not give an indication of the type of catalysis involved.

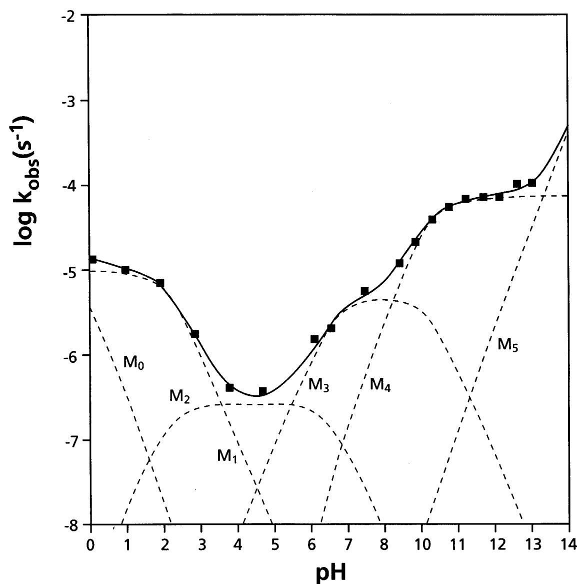

Fig. 2. pH-log k_{obs} profile at a temperature of 70°C and μ(NaCl) = 0.3.

Table 2
Macro reaction constants and maximum values of the corresponding individual reaction constants (log k)

Macro reaction constants		Log values of corresponding individual reaction constants
M_0	−5.4	$k_0^H = -5.4$
M_1	−5.0	$k_1^H = -2.8$ or $k_0^S = -5.0$
M_2	−8.8	$k_2^H = 0.4$ or $k_1^S = -6.6$ or $k_0^{OH} = 3.8$
M_3	−14.6	$k_3^H = 4.6$ or $k_2^S = -5.3$ or $k_1^{OH} = 0.27$
M_4	−23.4	$k_3^S = 4.2$ or $k_2^{OH} = -1.5$
M_5	−36.7	$k_3^{OH} = -4.8$

The minimum in the profile (see Fig. 2) indicates the maximum stability under the reaction conditions to be between pH 4 and 5. The inflection at pH 2 is caused by deprotonation of the dimethylamino group, consistent with a pK_a of 1.9 determined experimentally by measuring the absorption maximum at 255 nm as a function of the pH and with the calculated macro pK_a value of 2.3. Under alkaline conditions, the benzoylurea group will deprotonate. This causes additional inflections in the pH profile, the first one around pH 8.5, the second at pH 10.5, The calculated pK_a's are 7.0 and 10.0. A pK_a of 11.6 was found by the change in absorbance at 261 and 290 nm as a function of the pH. The differences between the calculated and the measured values may be a temperature effect (profile measured at 70°C, UV spectra at room temperature).

The pK_a of 8.5 could not be confirmed by UV: the relevant maxima are obscured by absorption bands of the substituted benzene rings. Deprotonations in the benzoylurea group do not seem to lead to an expanded chromophore and, thus, not to a red shift of one or more of the absorption maxima. This indicates that, due to the bulky dimethylamino group in *ortho* position in the one benzene ring, the molecule is not planar, thus reducing the mesomerism of the conjugated system.

3.5. Arrhenius plot

At fixed pH, k_{obs} is related to the temperature T according to the Arrhenius equation $\ln k_{obs} = \ln A - E_a/RT$, where A is the frequency factor, E_a the activation energy, T the temperature, and R the gas constant. An Arrhenius plot of the clanfenur degradation ($\ln k_{obs}$ vs. $1/T$) was constructed at pH 7. A linear relationship is observed (correlation coefficient 0.996). At pH 7, the activation energy and the frequency factor were calculated to be 114 kJ/mol and 4.4×10^{11}/s, respectively. The half-lives of clanfenur degradation at temperatures below 60°C were calculated by extrapolation of the Arrhenius plot. At pH 7, the half-lives were calculated to be 141 years and 9.5 years at 4°C and 25°C, respectively, well above the half-lives reported for the Cremophor EL®/ethanol formulation (Jonkman-de Vries et al., 1997). Obviously, Cremophor EL®/ethanol decreases the stability of clanfenur.

3.6. Effect of ionic strength and buffer concentration

The effect of the ionic strength and buffer concentration were determined in acidic, neutral, and alkaline solutions. Variations in the data are within experimental error.

3.7. Characterisation of degradation products

Jonkman-de Vries et al. (1997) identified *p*-chloroaniline (Fig. 1(5)) as one of the degradation products by photodiode-array detection and by the capacity factor in HPLC (peak 3 in Fig. 3). Although they obtained UV spectra of the other three degradation products (peak 1, 2 and 4), the degradation products could not be identified due to lack of reference samples.

We identified two of the remaining degradation products by LC-ESI-MS. Although the use of a 75-μm capillary cell in the on-line UV detector

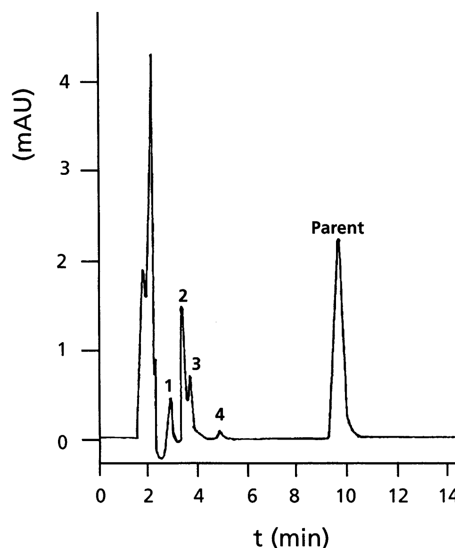

Fig. 3. Chromatogram of degraded clanfenur; clanfenur (parent), the degradation product *p*-chloroaniline (peak 3) and three additional degradation products (peaks 1, 2 and 4); the identity of 1,2 and 3 was established with LC-MS.

lowered the sensitivity in HPLC system II, the pattern of degradation products in the chromatograms could be recognised and masses assigned.

LC-MS spectra of acidic, neutral, and alkaline degradation samples, obtained in the positive ion mode, resulted in identification of peak 2 (Fig. 3) as *p*-chlorophenylurea ($[M+H]^+ = 171$, Fig. 1(4). The identity of peak 3 as *p*-chloroaniline was confirmed by its $[M+H]^+$ peak at m/z 127/129. $[M+H]^+$ of peak 1 was found to be 183, corresponding to 2-fluoro-6-dimethylaminobenzamide (Fig. 1(3)), an other likely degradation product (mass spectrum Fig. 4). During degradation, 2-fluoro-6-dimethylaminobenzoic acid and *p*-chlorobenzocarbamic acid are probably also formed. *p*-Chlorobenzocarbamic acid is unstable; it decarboxylates to *p*-chloroaniline. In both HPLC systems used, 2-fluoro-6-dimethylaminobenzoic acid is negatively charged and will not show retention. The structure of peak 4 has not been elucidated.

Loop injections of clanfenur were carried out in both the positive and negative ion mode. In the positive ion mode, fragmentation is influenced by the cone voltage. At a cone voltage of 20 V the base peak in the spectrum is a fragment ion with $m/z = 209$; a probable structure for this fragment ion is presented in Fig. 5. Ionisation at a higher cone voltage of 35 V results in two equally intense ions at $m/z = 209$ and 166, the latter probably being 2-fluoro-6-dimethylaminobenzoyl carbonium ion (Fig. 5). When a cone voltage of 50 V is applied, m/z 166 is the base peak.

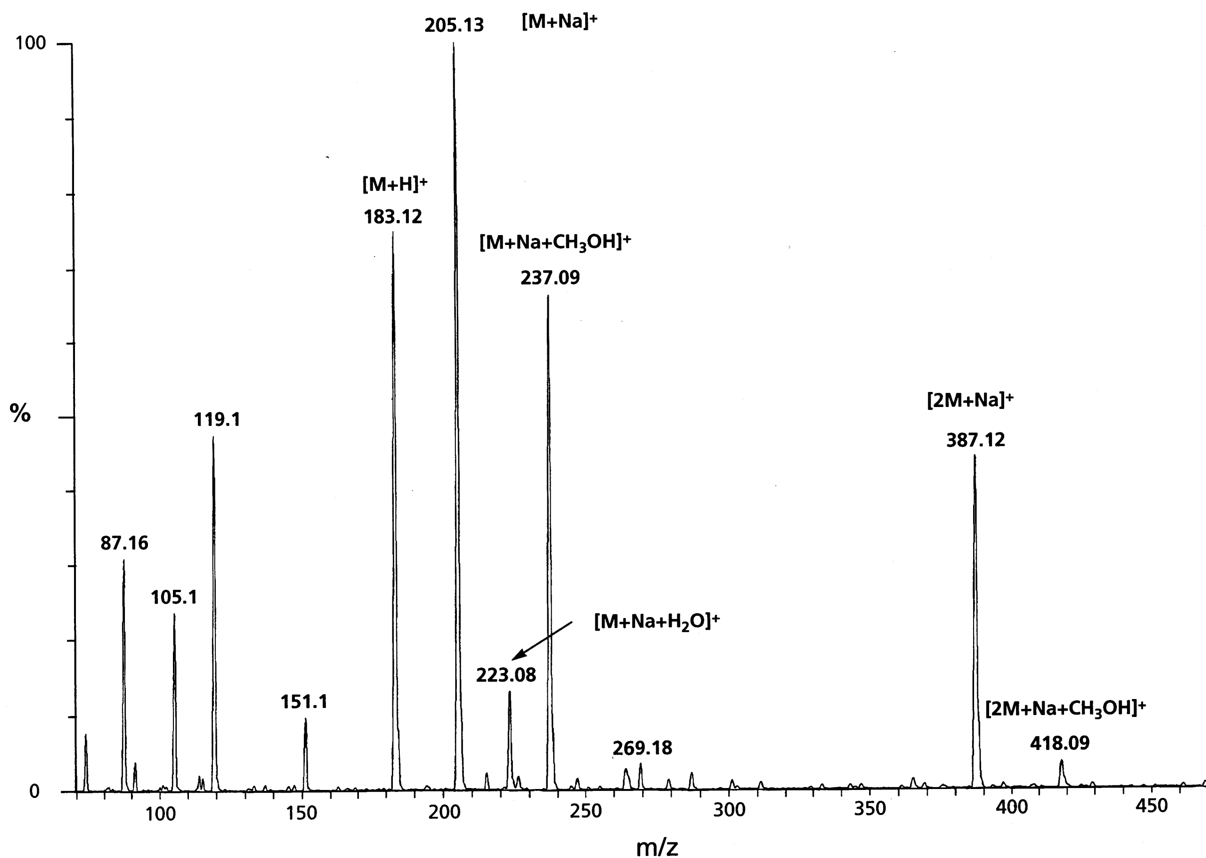

Fig. 4. Mass spectrum of peak 1 (alkaline degradation sample), showing the $[M+H]^+ = 183$ (2-fluoro-6-dimethylaminobenzamide).

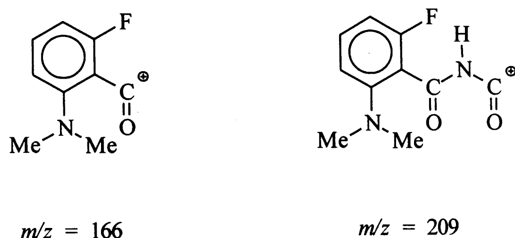

Fig. 5. Structures of the fragment ions with $m/z = 166$ (left) and 209 (right).

In the negative mode, the deprotonated molecule $[M\text{-}H]^-$ of clanfenur ($m/z = 335$) was observed in the spectrum after ionisation at a cone voltage of 120 and 200 V. No fragmentation was observed, not even at these high cone voltages.

4. Conclusions

The degradation of clanfenur in aqueous solution follows (pseudo-) first-order kinetics and can be described by a three-pK_a model. Degradation takes place by hydrolysis of the amide bonds. Between pH 4 and 5 the k_{obs} has its lowest value, indicating that clanfenur has its maximum stability in this pH range.

In the pH range 0–4, the degradation is most likely mainly acid-catalysed, in the pH range about 5–13 it is predominantly base-catalysed. The calculated pK_a's are 2.3, 7 and 10. Changes in ionic strength and buffer concentration do not affect the degradation of clanfenur in alkaline, neutral, and acidic media.

The degradation reaction at pH 7 satisfies Arrhenius' law over a temperature interval of 60–100°C. Extrapolation results in calculated half-lives of 141 years at 4°C, and 9.5 years 25°C.

So far, three degradation products were identified by LC-ESI-MS: *p*-chloroaniline, *p*-chlorophenyl urea, and 2-fluoro-6-dimethylaminobenzamide. An additional, likely degradation product, not yet confirmed by LC-MS, is 2-dimethylamino-6-fluorobenzoic acid.

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