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LC–UV method development and validation for the investigational anticancer agent imexon and identification of its degradation products

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Abstract

Imexon (4-imino-1,3-diazabicyclo[3,1,0]-hexan-2-one) is a member of the class of 2-cyanoaziridine derivatives, which have been of interest as immunomodulators and anticancer agents since the late 1970s. The pharmaceutical development of imexon necessitated the availability of an assay for the quantification and purity determination of imexon active pharmaceutical ingredient (API) and the drug in its pharmaceutical dosage form. A liquid chromatographic (LC) method with ultraviolet (UV) detection was developed, using a reverse phase column with phosphate buffer (pH 6; 50 mM) as mobile phase and UV detection at 230 nm. Although retention capacity for imexon was small (capacity factor of 0.5), the method was found to be linear over the concentration range of interest of 1.0–25 μg/mL, precise, accurate, and stability-indicating. Moreover, the use of LC–mass spectrometry (MS) and on-line photodiode array (PDA) detection enabled us to propose structures for four degradation products. Two of these products were also found as impurities in the API. The degradation products, including chloro- and hydroxy-derivatised products were shown to arise from nucleophilic reactions with the activated aziridine moiety of imexon. The developed LC–UV method was found suitable for the pharmaceutical quality control of imexon API and the drug in its pharmaceutical dosage form.

Keywords: Imexon; Reversed-phase liquid chromatography; Ring-destroying aziridine reactions; Mass spectrometry; Validation

1. Introduction

Imexon (4-imino-1,3-diazabicyclo[3,1,0]-hexan-2-one) is a member of the class of 2-cyanoaziridine derivatives, which have been of interest as immunomodulators and anticancer agents since the late 1970s [1]. Imexon was shown to be active in a variety of tumour cell lines and animal tumour models [2,3]. More recent in vitro research demonstrated higher cytotoxic activity for lymphoid malignancies, such as malignant lymphomas and multiple myeloma [2]. The proposed mechanism of action for imexon-induced cytotoxicity suggests a sequential pathway initiated by binding of the aziridine moiety of imexon to sulfhydryl groups of cysteine residues, resulting in decreased levels of cellular thiols in myeloma cells. Cellular thiols protect the cell from oxidative damage generated by reactive oxygen species (ROS). Compromising the antioxidant defence systems results in accumulation of ROS in the mitochondria, leading to oxidative stress and the induction of apoptosis [4,5]. Imexon does not appear to be a DNA alkylator.

The pharmaceutical development of a stable, sterile, injectable dosage form for the scheduled phase I clinical trials in patients with myeloma and other refractory cancer necessitated the availability of a validated assay [6,7] for the quantification and purity determination of imexon active pharmaceutical ingredient (API) and the drug in its pharmaceutical dosage form. The development of a reversed-phase liquid chromatography (RPLC) method with ultraviolet detection (UV) for this purpose is described in this paper. Prior

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to the assay development, imexon was structurally characterised using mass spectrometry (MS), nuclear magnetic resonance (NMR), X-ray diffraction (XRD), and infrared (IR) spectroscopy. Validation of the analytical method was performed according to international guidelines [8,9]. On-line photodiode array (PDA) detection and LC coupled to MS were used to gain more information on the observed impurities and degradation products of imexon.

2. Materials and methods

2.1. Chemicals

Imexon (Mw 111), BM41.209 (Mw 111), and BM41.237 (Mw 112) were provided by Heidelberg Pharma GmbH (Ladenburg, Germany). Imexon injectable product was manufactured in-house (Department of Pharmacy and Pharmacology, Slotervaart Hospital, Amsterdam, The Netherlands) by freeze-drying a dimethyl sulfoxide (DMSO) solution containing imexon, polysorbate 80, and polyvinylpyrollidone (PVP; Kollidon 12 PF). Water for chromatography, disodium hydrogen phosphate, and sodium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade and were used without further purification.

2.2. Infrared spectroscopy

IR spectra (400–4000 cm^{-1}) were obtained with a Model PU 9706 IR spectrophotometer (Philips Nederland B.V., Eindhoven, The Netherlands) using the potassium bromide (KBr) pellet technique. The pellet consisted of 2 mg imexon drug substance and 100 mg KBr. The ratio-recording mode was auto-smooth and the scan time 8 min.

2.3. Nuclear magnetic resonance analysis

The ^{1}H NMR (^{1}D and HH-COSY) and ^{13}C NMR (attached proton test, APT) were recorded with a Gemini 300 BB instrument (Varian Assoc., Palo Alto, CA, USA) operating at 300.1 MHz for ^{1}H and 75.5 MHz for ^{13}C. Imexon drug substance was dissolved in DMSO-d_6 to a concentration of approximately 10 mg/mL. In the ^{1}H spectrum, the central DMSO line at δ 2.505 ppm was used as the internal reference; in the ^{13}C spectrum, the centre line in the DMSO signal at δ 39.7 ppm.

2.4. X-ray diffraction

XRD was performed with a Model PW 3710 PC-APD diffractometer (Philips, The Netherlands) at atmospheric humidity over a 2θ range of 5–40° (where theta is the scattering angle). Samples (0.2–0.5 g) were placed in open rectangular aluminium sample holders (100 mm × 80 mm). The Cu Kα radiation from the anode operating at 40 kV and 50 mA was monochromised with a 15 μm Ni foil. Scan step size was 0.02° and steptime 4.0 s.

2.5. Sample preparation

2.5.1. Imexon reference standard solution

Imexon reference standard solution was prepared by accurately weighing approximately 1.0 mg reference standard and dissolving it in 50 mL of phosphate buffer (pH 7.4; 20 mM), to obtain a final concentration of 20 μg/mL imexon. The reference standard solution was stored at 2–8 °C for a maximum of 24 h until analysis.

2.5.2. Imexon calibration and quality control samples

Imexon stock solutions were prepared by accurately weighing 1.0 mg API and subsequent dilution in 50 mL of phosphate buffer (pH 7.4; 20 mM), corresponding to 50 μg/mL imexon. Two independent stock solutions were used to prepare calibration and quality control samples. Calibration samples were prepared at concentration of 1.0, 2.5, 5.0, 10, 15, and 25 μg/mL imexon and quality control samples at 5.0, 10, and 20 μg/mL imexon. To obtain sample solutions of 1.0, 2.5, 5.0, 10, 15, 20, and 25 μg/mL imexon, subsequently 20, 50, 100, 200, 300, 400 and 500 μL of stock solutions were transferred to an autosampler vial. Phosphate buffer (pH 7.4; 20 mM) was added to a final volume of 1000 μL. Samples were stored for a maximum of 24 h at 2–8 °C until analysis.

2.5.3. Imexon API

Imexon drug substance sample was prepared by accurately weighing 1.0 mg of API and subsequent dissolution in 50 mL of phosphate buffer (pH 7.4; 20 mM), corresponding to 20 μg/mL imexon. Samples were analysed directly after preparation.

2.5.4. Pharmaceutical dosage form

Imexon 100 mg/vial lyophilised powder for intravenous use was dissolved in 10 mL of phosphate buffer (pH 7.4; 20 mM). One hundred microliters of the resulting solution was diluted to 10 mL with the same solvent. Two hundred microliters of it was further transferred to an autosampler vial containing 800 μL of the same solvent. Samples were analysed directly after preparation.

2.6. Liquid chromatography

The HPLC system consisted of an 1100 series binary HPLC pump, model G1312A (Agilent Technologies, Amstelveen, The Netherlands), a SpectraSERIES AS3000 automatic sample injection device, equipped with a 100 μL sample loop (Thermo Separation Products, Breda, The Netherlands), and a 996 photodiode array detector (Waters Chromatography B.V., Etten-Leur, The Netherlands). Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA). Separation was achieved using a Zorbax Bonus RP analytical

column (150 mm × 4.6 mm i.d., particle size 5 μm, Rockland Technologies Inc., Newport, DE, USA), which was protected by a guard column (10 mm × 3 mm i.d.) packed with reversed-phase material (Chrompack, Middelburg, The Netherlands).

The mobile phase, pumped at a flow rate of 0.8 mL/min, consisted of phosphate buffer (pH 6; 50 mM). Sample volumes of 10 μL were injected and a run time of 20 min was employed. UV-detection was performed at 230 nm.

2.7. Validation procedure

The LC method was validated with respect to the following parameters: linearity, accuracy, precision, selectivity and stability-indicating capability, and stability of reference standard solutions [8,9].

2.7.1. Linearity

Calibration curves at six concentration levels (1.0, 2.5, 5.0, 10, 15, and 25 μg/mL imexon in phosphate buffer (pH 7.4; 20 mM)) were analysed in duplicate in three separate runs. Least-squares analysis of concentration, weighted by [1/concentration], versus the area of the imexon peak was applied. The linearity of the calibration curves was evaluated by means of back-calculated values of the calibration standards, the deviation in response factors at different concentration levels and the observed correlation coefficients of the calibration curves [10].

2.7.2. Accuracy and precision

Accuracy, and within-run and between-run precision of the method were determined by assaying quality control samples at three concentration levels (2.5, 10 and 20 μg/mL imexon in phosphate buffer (pH 7.4; 20 mM)) in triplicate in three separate analytical runs. Accuracy was measured as the percent deviation from the nominal concentration. The within- and between-run precisions were calculated by analysis of variance (ANOVA) for each test concentration using the analytical run as the grouping variable. From the ANOVA analysis the day mean square (DayMS), error mean square (ErrMS) and grand mean (GM) were obtained. Within- and between-run precisions were defined using Eqs. (1) and (2) [10], respectively, where n is the number of replicates:

$$\text{within-run precision (\%)} = 100 \times \frac{\sqrt{\text{ErrMS}}}{\text{GM}} \quad (1)$$

$$\text{between-run precision (\%)} = 100 \times \frac{\sqrt{(\text{DayMS} - \text{ErrMS})/n}}{\text{GM}} \quad (2)$$

2.7.3. Selectivity and stability-indicating capability

Selectivity was tested in the presence of the excipients polysorbate 80 and PVP, and the residual solvent DMSO present in the pharmaceutical dosage form.

The stability-indicating capability of the LC method was tested by subjecting imexon stock solutions in the concentration of 10 mg/mL imexon in water to several stress conditions for 1 h (oxidation) or 24 h (acid, alkaline, and heat) [10]. Heat: 1 mL of imexon stock solution was exposed to 100 °C. Oxidation: to 1 mL of imexon stock solution, 1 mL of a 30% hydrogen peroxide solution was added. Acidic: to 1 mL imexon solution 0.5 mL 1 M hydrochloric acid (HCl) was added. Samples were neutralised using 1 M sodium hydroxide (NaOH). Alkaline: similar to the method described under "acidic", using 1 M NaOH as alkalizer and 1 M HCl to neutralise the sample solution. All samples were diluted with phosphate buffer (pH 7.4; 20 mM) to a theoretical concentration of 20 μg/mL imexon before analysis.

2.7.4. Stability of reference standard solutions

Reference standard solutions were stored at ambient temperature (20–25 °C) and light, at refrigerated (2–8 °C) temperatures and at −20 °C, in the dark. Imexon content and purity were monitored in time.

2.8. Liquid chromatography/mass spectrometry (LC/MS)

The mobile phase used for the LC/MS experiments was pure water. The LC system consisted of an HP1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) with a binary pump, autosampler, and degasser. Other LC conditions were as described above. The eluate was led into the interface of an API 2000 triple quadrupole MS equipped with an electrospray ionisation (ESI) source (Sciex, Thornhill, Ont., Canada). The MS was operated in the positive ion mode. The ion spray voltage was 5500 V and the source temperature was set at 450 °C. A range of m/z 30–250 amu was scanned for the identification of unknown degradation products, using a stepsize of 0.10 amu with dwell times of 3 s. The nebulizer gas (compressed air), turbo gas (compressed air), curtain gas (N_2), and collision activated dissociation gas (N_2) were operated at 40, 80, 30, and 2 psi, respectively.

3. Results and discussion

3.1. Characterisation of imexon drug substance

Imexon reference standard lot 90008-92 was structurally characterised by MS, IR, ^{1}H and ^{13}C NMR, and XRD analysis. The MS and MS/MS data, which were in agreement with the assigned structure without conclusive evidence of the presence of an aziridine ring, are further discussed in the section "stability-indicating capability". In the IR spectrum of imexon many characteristic absorption bands for the aziridine ring were observed, of which most could be ascribed to the vibrations of the CH_2 present in the constrained ring [11]. Furthermore, amide bands (I–III) were observed and O–H stretching and C–O bending absorption bands were present as a result of keto-enol tautomerisation. These and

Table 1
Structural characterisation of imexon reference standard lot 90008-92

Analytical method	Results
IR analysis (characteristic absorption bands (approximately), cm^{-1})	3230 (N—H stretching and O—H stretching); 3080 and 3000 (CH_2 stretching); 1730–1640 (amide I band); 1550–1400 (amide II band and CH_2 deformation); 1290 (amide III band); 1230 (CH_2 twisting); 1170 (C—O stretching); 1128 and 1007 (CH_2 deformation (wagging)); 1100 (CH_2 twisting); 855 (symmetrical aziridine ring deformation); 812 (CH_2 rocking)
^{1}H NMR analysis, δ (ppm)	2.23 (d, 1, J = 3 Hz, HCH); 2.44 (d, 1, J = 6 Hz, HCH); 3.35 (s, HOD); 3.36 (dd, 1, J = 6/3 Hz, CH); 8.73 (s, 2, NH)
^{13}C NMR analysis, δ (ppm)	38.3 (tertiary, CH); 44.4 (secondary, CH_2); 183.0 (quaternary, CO)
XRD (distinct refraction peaks at 2θ) (°)	15.9, 17.4, 18.8, 21.5, 22.0, 24.3, 24.9, 26.7, 28.2, 29.5, 31.5, 34.6, 36.1, 37.0, and 38.1

other major assignments are given in Table 1. The NMR data (Table 1) confirmed the MS and IR data, although the ^{13}C signal of the C=N carbon was lost in the noise, due to the absence of a nuclear overhauser effect (NOE; quaternary carbon) and its double bond with the nitrogen. The presence of several distinct peaks in the XRD diffraction patterns of imexon indicated that imexon is present as crystalline material.

3.2. LC–UV method development

Imexon is a polar molecule, initially directing LC development toward normal phase LC. The normal phase LC method described in literature for the analysis of imexon in mice and dog plasma, however, showed limited retention (retention time of 3.5 min) of imexon while using a mobile phase containing only 5% water [3]. Besides quantification of imexon, determination of possible degradation products and impurities is of importance during the development of a pharmaceutical dosage form. The two known impurities originating from the manufacture of imexon API (BM41.209 and BM41.237) (Fig. 1) would exhibit even less retention when employing normal phase chromatography. The use of gas chromatography for the analysis of the small molecule imexon was not pursued as the drug appeared to be very sensitive to degradation upon exposure to heat. To analyse imexon together with its impurities and possible degradation products, reversed phase LC in combination with ultraviolet (UV) detection was tested. The only absorption maximum present in the UV-spectrum of imexon at a wavelength of 230 nm was selected for UV detection. Using a Zorbax Bonus RP column and a mobile phase comprised of 100% aqueous phase buffered with sodium phosphate (pH 6; 50 mM), the neutral molecule imexon was retained for 1.1 min (k' 0.5; dead volume of 2.2 min) and the impurities BM41.209 and BM41.237 for 4.0 min (k' 1.7) and 7.7 min (k' 3.4), respectively. This system was tested for linearity of the concentration response curve for imexon. The initial concentration range of 25–300 μg/mL imexon, however, resulted in non-linear concentration response curves. Moreover, substantial carry-over was observed. Decreasing the imexon concentrations to 1–25 μg/mL eliminated both problems and resulted in acceptable peak shape (asymmetry ≤ 1.3, theoretical plates > 1400). A representative chromatogram of imexon 20 μg/mL is given in Fig. 2.

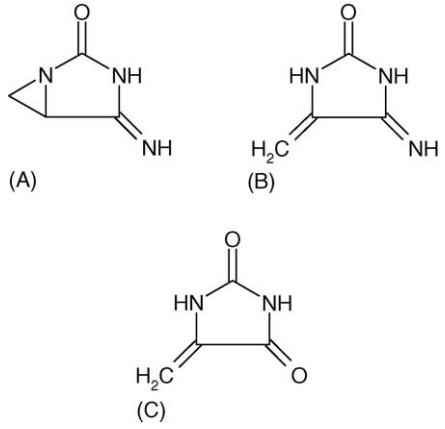

Fig. 1. Chemical structure of imexon ($C_4H_5N_3O$, Mw = 111) (A), BM41.209 ($C_4H_5N_3O$, Mw = 111) (B), and BM41.237 ($C_4H_4N_2O_2$, Mw = 112) (C).

3.3. Validation

3.3.1. Linearity, accuracy and precision

Linearity, accuracy, within- and between-run precisions of the LC–UV method were examined over the concentration range of 1.0–25 μg/mL. This range corresponds to 5–125% of the intended test concentration of 20 μg/mL for the pharmaceutical quality control of imexon API and the drug in its

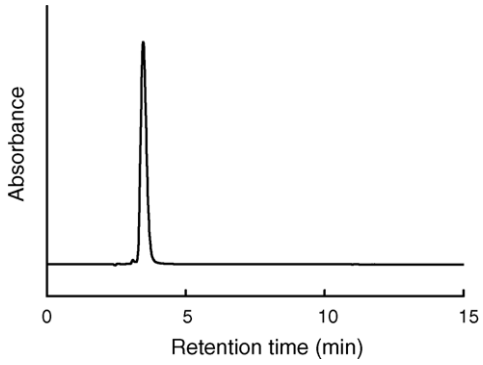

Fig. 2. Representative chromatogram of 20 μg/mL imexon drug substance solution. The retention time of imexon is approximately 3.4 min (k' 0.5). Detection: 230 nm.

Table 2
Results of imexon quality control samples ($n = 3$ per concentration level)

Concentration (nominal, μg/mL)	Within-run precision (%)	Between-run precision (%)	Accuracy (%)
2.56	1.7	n.a.	100.8
10.2	0.87	1.3	99.70
20.5	1.1	0.83	100.5

n.a.: no significant additional variation due to the performance of the assay in different runs.

Table 3
Relative peak areas (%) of imexon, impurities and degradation products (numbered 1–5) in imexon API lot 44886601 and after exposure of this lot of API in solution to heat, acidic, and alkaline conditions for 30 min and oxidation for 10 min, compared to imexon reference standard lot 90008-92

Product	rrt	Reference standard	API	Acidic	Alkaline	Heat	Oxidation
1	0.83	0.1	0.1	5.5	0.7	10.2	0.9
Imexon	1.0	99.8	99.8	0.0	36.0	14.7	23.3
2	1.2	–	–	–	0.6	–	1.7
3	1.3	–	–	4.4	0.3	–	–
4 (BM41.209)	1.9	0.1	0.1	33.7	0.5	6.3	0.2
5 (BM41.237)	3.0	–	–	0.1	–	0.2	–

rrt: relative retention time.

pharmaceutical dosage form. The assay was linear with a relative standard deviation of 1.1% in the response factors (area divided by concentration) obtained in the tested concentration range and correlation coefficients were ≥0.9999 for all three calibration curves. The average back-calculated concentration for the different calibration concentration levels in the six analytical runs varied between 98.7% and 101.2% of the theoretical concentration. The maximal deviation from the nominal concentration was 3.0%, which was found at the lowest calibration level. Within- and between-run precisions were ≤1.7% with accuracies between 99.70% and 100.8% (Table 2).

3.3.2. Stability of the reference standard solution

Degradation of imexon in the reference standard solutions was observed within 4 h of storage at ambient light and temperature (20–25 °C). Although areas of 98.2 ± 1.7% of the initial peak area were still present, chromatographic purity decreased from 99.3% to 98.3%. Increasing peak area percentages were observed at relative retention times (rrt) of 0.8 and 1.9, corresponding to products 1 and 4 observed during accelerated stress testing (see "stability-indicating capability" and Table 3). Although stability was increased at a storage condition of 2–8 °C in the dark, increased peak area percentages of products 1 and 4 were still observed after 4 h of storage. At −20 °C, only 49.6% of the initial imexon peak area was recovered after 24 h of storage, compared to 90.9 ± 0.9% and 98.5 ± 1.7% after 24 h of storage at ambient and refrigerated temperature, respectively. Chromatographic purity decreased to 84.2%. Probably responsible for the increased degradation of imexon at −20 °C is the difference in solubility between the disodium hydrogen phophate and sodium dihydrogen phosphate present in the imexon standard solution causing a pH drop in the solution upon freezing [12].

As a consequence of the observed low stability, imexon reference standard solutions could be stored for a maximum of 24 h at 2–8 °C with no significant decrease in imexon peak area (98.5 ± 1.7%) and sample preparations were analysed directly after preparation to be able to determine imexon purity.

3.3.3. Stability-indicating capability

Imexon is pharmaceutically formulated as a lyophilised dosage form containing the excipients polysorbate 80, PVP and the residual solvent DMSO (<6%). These compounds were added to imexon quality control samples in a concentration corresponding to the imexon to compound ratio present in the formulation. The compounds were not recovered in the chromatogram and did not affect the response of imexon.

Fig. 3 shows the percentage imexon remaining with respect to the initial concentration at several time points under the different stress conditions. Fast degradation was observed for all the solutions with undetectable imexon concentrations remaining after 24 h of storage. The highest degradation rate was observed under acidic conditions. Imexon was more stable after exposure to alkaline conditions and after expo-

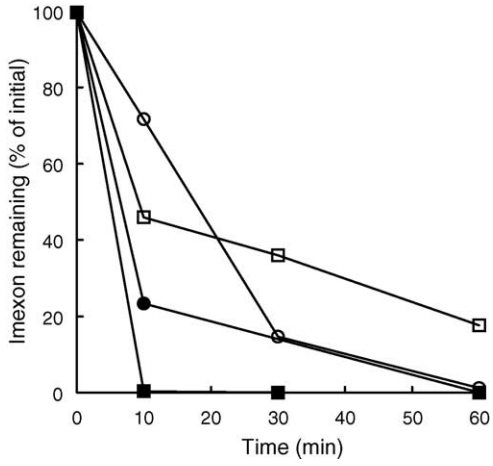

Fig. 3. Degradation of imexon under different stress conditions (◯, heat; □, alkaline; ●, oxidation; ■, acid).

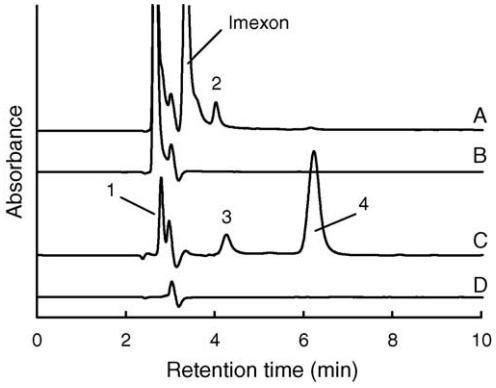

Fig. 4. Degradation chromatograms of imexon exposed to oxidation with hydrogen peroxide (A) and to acid with HCl (C) for 10 min with the corresponding blank injections of hydrogen peroxide in water (B) and HCl in water (D). The numbers correspond to the products given in Tables 2 and 3.

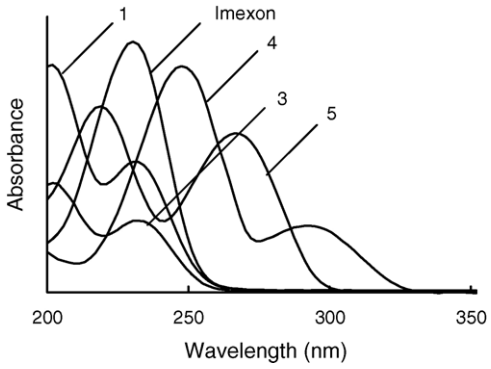

Fig. 5. UV spectra of imexon and the degradation products 1, 3–5. The numbers correspond to the products given in Tables 2 and 3, and Fig. 3.

sure to heat (100 °C) at neutral pH. Table 3 lists the relative peak areas of imexon and additional impurities or degradation products observed after exposure to the tested conditions for 30 min or 10 min (oxidation). Additional products were only included in the table when present at relative peak areas of at least 0.2% in one of the analysed samples. Several degradation peaks were observed and some existing impurities increased. The degradation into product 5 was only minor after 30 min but was shown to increase with time to 5.4% of the initial imexon peak area after 24 h of exposure to acidic conditions. The additional peaks were well separated from the parent peak, which is illustrated by the chromatograms obtained after exposure to oxidation (Fig. 4A) and acidic conditions (Fig. 4C) for 10 min. Products 1 and 4 were also observed in the chromatogram of imexon API lot 44886601 and reference standard lot 90008-92 in phosphate buffer (pH 7.4; 20 mM) (Table 3). Both products were more abundantly present after exposure to one or more of the stress conditions, indicating that these were degradation products of imexon.

The UV-spectra recorded for imexon and the observed additional peaks during the forced degradation study with on-line PDA detection are given in Fig. 5 and the absorption maxima in Table 4. No absorption maxima could be recorded for product 2 probably due to the small amounts formed. The UV spectra obtained with on-line PDA detection of the additional peaks clearly deviate from the imexon spectrum, indicating modifications in the chromophore of the molecule. The UV-spectra of products 1 and 3 are similar, suggesting modifications of the same kind in the imexon molecular structure. The UV spectra of products 4 and 5 were identical to the UV spectra obtained for the known impurities BM41.209 and BM41.237 (Fig. 1), respectively. The same was true for the obtained retention times, with coinciding peaks observed when analysing degraded samples spiked with BM41.209 and BM41.237.

The UV-spectrum of the imexon peak remained identical during degradation at the different stress conditions and no additional ions were observed with LC–MS analysis. This indicates that there were no degradation products co-eluting with imexon, and demonstrates the stability-indicating capability of the analytical method.

To confirm the structures of product 4 and 5 to be BM41.209 and BM41.237 and to elucidate the structures of the other degradation products observed during the forced degradation studies, the LC system was coupled to MS. For MS detection, buffer components present in the mobile phase should be preferably volatile in order to prevent signal suppression and pollution of the MS interface and quadrupoles. Therefore, the mobile phase containing 50 mM phosphate buffer used for LC–UV analysis was replaced by pure water. The chromatogram obtained after injection of imexon reference standard solution was similar to the chromatogram obtained with the phosphate buffer with respect to retention and peak shape. Moreover, the retention times and UV-spectra (obtained with on-line PDA detection) of the degradation peaks generated during stress testing were similar to those observed using phosphate buffered mobile phase.

A solution of 10 μg/mL of imexon in water was continuously infused (0.5 mL/h) into the ESI source and mass spectra were recorded in the first quadrupole (Q1) of the

Table 4
MS and UV data of imexon and degradation products numbered 1 to 5 (see also Fig. 4)

Product	*m/z* values MS $[M+H]^+$	*m/z* values MS/MS	UV maxima (nm)	Proposed structure
1	130	103, 100, 99, 87, 69, 59	202, 230	4-Hydroxymethyl-5-imino-imidazolidin-2-one
Imexon	112	95, 84, 71, 69, 42	230	4-Imino-1,3-diazo-bicyclo[3,1,0]hexan-2-one
2	–	–	–	?
3	148	131, 121, 105, 99, 78, 69	202, 232	4-Chloromethyl-5-imino-imidazolidin-2-one
4 (BM41.209)	112	95, 85, 69, 68, 42	248, 291	4-Imino-5-methylene-imidazolidin-2-one
5 (BM41.237)	–	–	219, 267	5-Methylene-imidazolidin-2,4-dione

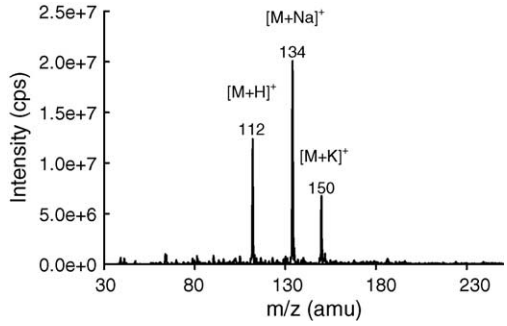

Fig. 6. Positive Q1 spectrum of imexon using continuous infusion.

MS (Fig. 6). The $[M+Na]^+$ ion of imexon (*m*/*z* 134) was most abundantly present in the spectrum, followed by the $[M+H]^+$ and $[M+K]^+$ ions (*m*/*z* 112 and 150, respectively). As $[M+H]^+$ ions are more readily fragmented than sodium adducts [13], MS/MS analysis was performed by selecting the *m*/*z* value for the molecular ion and inducing it to fragment in the collision cell using nitrogen gas, after which the fragments were detected in the third quadrupole of the MS. For imexon and the degradation products susceptible to positive ionisation, Table 4 lists the most abundant molecular ions recorded in the first quadrupole (Q1) and their corresponding MS/MS fragments observed in the third quadrupole. For each molecular ion, corresponding sodium adducts were observed in the Q1 scan. The observed molecular ion of *m*/*z* 129 for product 1 corresponded to an additional 18 amu compared to imexon, which is best explained by hydrolysis of imexon. The Q1 spectrum of product 3 clearly demonstrated additional peaks for the $[M+H]^+$ (*m*/*z* 148) and $[M+Na]^+$ (*m*/*z* 170) ion at plus 2 amu with a relative intensity of approximately 30%, indicating the presence of a chloric atom in the molecular structure. This product was observed after acid degradation using 1 N HCl. No degradation to product 3 was observed after repeating the exposure to acid using 1N nitric acid. The molecular ion observed for product 4 was equal to that observed for imexon ($[M+H]^+$ *m*/*z* 112). Ionisation of product 2 and 5 was not accomplished.

The product ion spectra of imexon and its degradation products susceptible to ionisation are given in Fig. 7. Product 4 (P4) and BM41.209 were both induced in the collision cell and similar fragmentation patterns (Fig. 7) were observed, confirming the identity of product 4 to be BM41.209. The most striking difference between the product ion spectra of imexon and BM41.209 compared to products 1 and 3 was the absence of fragment *m*/*z* 42 for products 1 and 3 and the presence of fragment *m*/*z* 99. For product 3, the $[M+H]^+$ ion and its chloric isotope were separately fragmented and fragment *m*/*z* 99 was shown to contain no chloride. The corresponding leaving group of *m*/*z* 49 could therefore only be assigned to the radical fragment CH_2–Cl, resulting in the structure proposal for product 3 (P3) depicted in Fig. 7. The similarities between the UV spectra of products 1 and 3 (Fig. 5) and the presence of fragments *m*/*z* 99 and 100 in the product ion scan of product 1 resulted in the structural proposal for product 1 (P1; Fig. 7). Although product 5 was not susceptible to ionisation and, therefore, confirmation of its identity could not be achieved, BM41.237 remained the most probable structure considering the identical UV-spectra and retention times observed and is therefore given as structural proposal for product 5 (P5) in Fig. 7.

The chemical structures of the proposed structures are given in Table 4.

3.3.4. Degradation mechanism

Imexon is an aziridine-containing compound. Ring-destroying reactions are known to occur readily in compounds with a so-called activated aziridine group containing a nitrogen substituent able to conjugate with the unshared electrons of the nitrogen [14]. For imexon, the carbonyl function is such a substituent. The ring-destroying reactions are initiated by protonation of the aziridine, which then becomes the primary target of nucleophilic attack by water or other nucleophiles. Fig. 8 demonstrates the reaction scheme proposed for imexon. Degradation product 1 is the result of the nucleophilic attack of water or hydroxyl on the aziridine ring. In the presence of hydrogen halides, ring-destroying reactions of activated aziridines are known to result in conjugation of the halide to the aziridine [14–16]. Degradation product 3 of imexon, identified as a chloro-derivative, was formed in the presence of hydrochloric acid at low pH. Chloro-derivatisation of aziridine containing compounds is, however, not only dependent on low pH. This is illustrated by the degradation of the aziridine compound tioTEPA with increasing degradation observed at acidic and neutral conditions (but not at alkaline conditions) in the presence of sodium chloride through the formation of chloro-derivatives [16]. For imexon, the chloro-derivative product 3 was also observed in the alkaline samples, which were titrated to neutral pH using hydrochloric acid. The hydroxy-derivative of imexon (product 1) was also formed in neutral (100 °C) and to a much lesser extent in alkaline conditions (Table 3). Although protonation of the aziridine nitrogen will still occur at neutral pH, the nucleophilic reaction under alkaline conditions indicates that protonation of the nitrogen is not necessary for the nucleophilic reaction with hydroxyl to occur, as also described for tioTEPA and other activated aziridines [14,16]. The most abundant degradation product present for imexon was BM41.209 (product 4) resulting from ring opening without further derivatisation. Subsequent oxidation of BM41.209 to BM41.237 (product 5) is probably proton catalysed as it was shown to increase in time under exposure to acid. With the exception of degradation product BM41.237, a decrease in percentages of the degradation products was observed with time. Total disappearance of degradation products was observed after 24 h of exposure to heat and alkaline conditions. This indicates that the identified degradation products of imexon are unstable and probably degrade to small molecule fragments not detected with the developed analytical method.

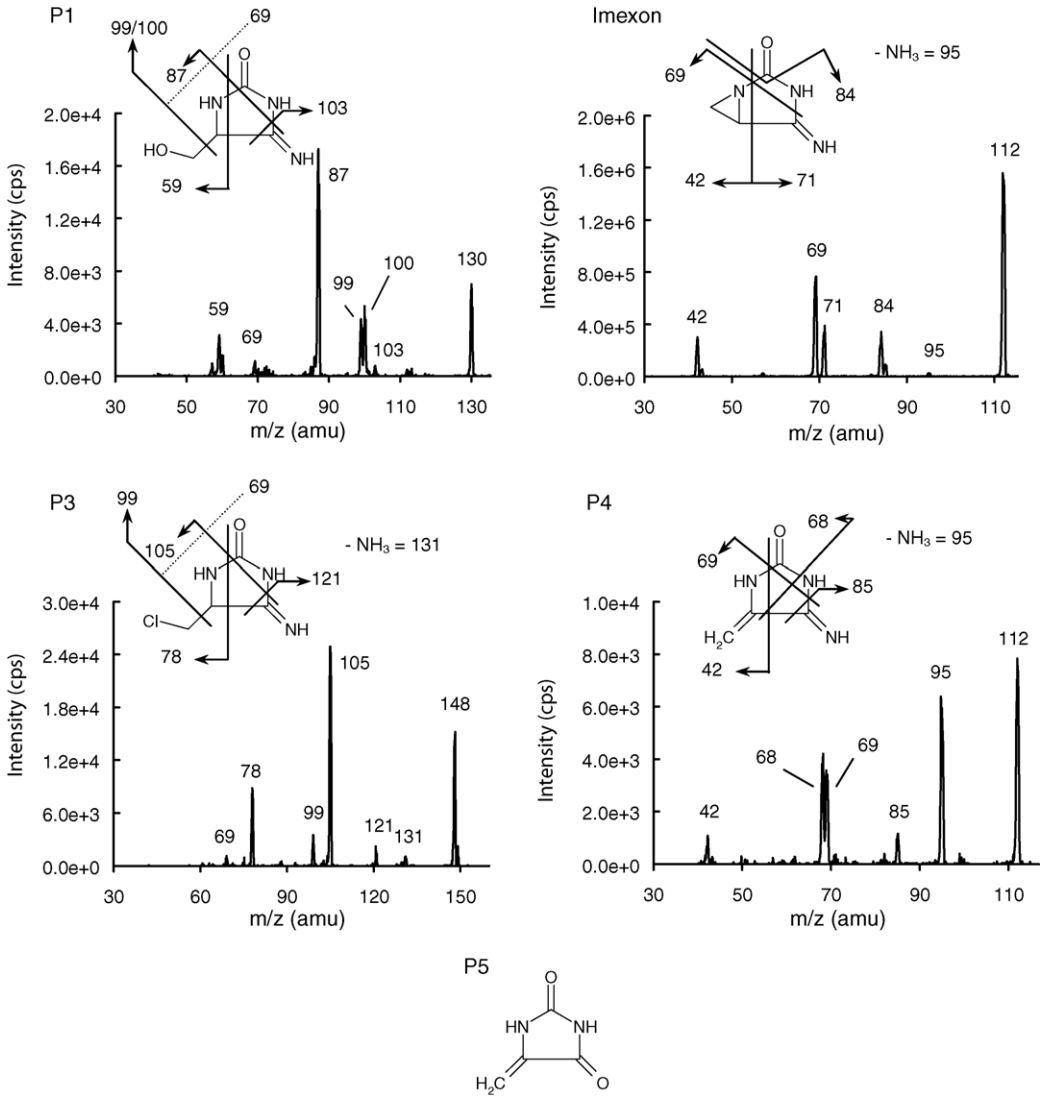

Fig. 7. Positive MS/MS spectrum of imexon and degradation products 1, 3, and 4 and structural proposals for products 1, 3–5. The molecular ions $[M+H]^+$ were selected for MS/MS analysis.

3.3.5. System suitability parameters

The observed linearity of the analytical method supports the use of a single reference standard concentration for the standardised quantitative analysis of imexon. The system suitability will consist of one blank injection (mobile phase), six injections of the imexon reference standard solution and one injection of a separately weighed imexon reference standard solution. Criteria for capacity factor, theoretical plates, tailing factor, %R.S.D. in area (six injections), and ratio between the area of the separately weighed reference standard solutions were defined as: $0.4 \leq k' \leq 1.0$, $N > 1400$, $0.8 \leq T \leq 1.5$, %R.S.D. < 1.0%, ratio 0.98–1.02, respectively.

The criteria for capacity factor were deduced from the minimal retention required for imexon to be able to detect degradation product 1 and the maximal retention to be able to detect late eluting degradation products within the run time of 15 min. The imexon content of sample solutions will be calculated using the bracket mode, in which the areas of two preceding and two following reference standard solutions are used.

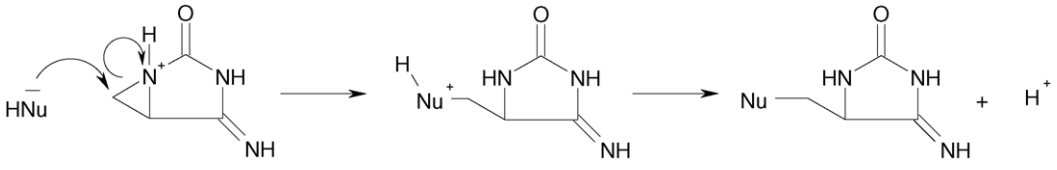

Fig. 8. Ring opening reactions of imexon with a nucleophilic reagent.

4. Conclusion

In conclusion, a stability-indicating analytical method comprising of separation with RPLC and subsequent UV detection was developed for the novel investigational anti-cancer agent imexon. The response of the method was found to be linear in the range of 1.0–25 μg/mL, and it proved to be precise and accurate. Although only minor retention of imexon was achieved, accelerated stress testing showed that all degradation products were well separated from the parent compound with unchanged UV and MS spectra observed for the imexon peak, confirming its stability-indicating capability. Moreover, the use of LC–MS and UV spectra enabled us to propose structures for four degradation products. Two of these products were also found as impurities in the API. The degradation products, including chloro- and hydroxy-derivatised products were shown to arise from nucleophilic reactions with the activated aziridine moiety of imexon. The developed stability-indicating LC–UV method was found suitable for the pharmaceutical quality control of imexon API and its pharmaceutical dosage form.

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