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Determination of diminazene aceturate in pharmaceutical formulations by HPLC and identification of related substances by LC/MS

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Abstract

A validated, reversed-phase, isocratic high-performance liquid chromatographic method for the simultaneous assay of diminazene aceturate, antipyrine (excipient) and diminazene impurities in pharmaceutical formulations is described. The chromatographic system consisted of a Lichrospher-60 RP-select B column with a mobile phase composition of acetonitrile–methanol–ammonium formate (pH 4.0, 20 mM) (10:10: 80 v/v/v) and UV detection at 254 nm. The method is specific, precise and accurate for the determination of diminazene in the presence of its manufacturing and degradation impurities with a limit of detection and quantification of 50 ng/ml and 10 μg/ml (RSD < 3.0%), respectively. The major manufacturing impurity [1-(4 amidino phenyl)3-(4 carbamoyl phenyl)-triazene] and a degradant (*p*-aminobenzamidine) of diminazene aceturate have been resolved and identified by liquid chromatography/electrospray ionization-mass spectrometry operated in a positive ion mode.

Keywords: Diminazene aceturate; Impurities; Amidino; Isocratic; Reversed-phase liquid chromatography; Electrospray ionisation mass spectrometry; NMR spectroscopy

1. Introduction

Diminazene aceturate (4,4′-diamidinodiazoaminobenzene diaceturate tetrahydrate) (Fig. 1) is an aromatic diamidine used extensively as a veterinary trypanocide and babesiacide [1,2] in affected areas of the world. Although not licensed for human use, it has been successfully employed in the treatment of early stage cases of human African sleeping sickness [3]. However, its efficacy has been curtailed by widespread drug resistance [4]. Despite its use for over four decades, there are no pharmacopoieal specifications for the quality control of the product. The expiry of patent protection of the innovator product (Berenil®)

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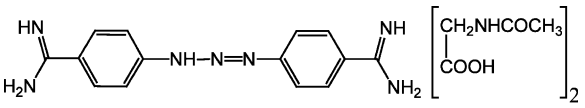
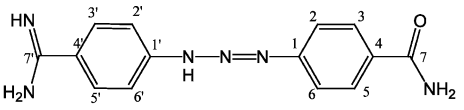
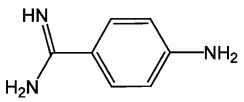


Diminazene diaceturate (I)



Impurity (II)



Degradant (III)

Fig. 1. Chemical structures of diminazene diaceturate (I), impurity (II) and degradant (III).

and the introduction of generic formulations necessitates the development of quality control techniques to safeguard the quality and subsequently the efficacy of the product.

The assay of diminazene in biological fluids by liquid chromatography has been associated with poor peak shape (tailing peaks), the use of complex mobile phases and poor recoveries [5,6]. The presence of two highly basic amidino groups (pKa 11) in diminazene (Fig. 1) makes it very susceptible to residual interactions with silanol groups of standard silica-based reversed phase stationary phases. Adsorption of basic compounds such as amidines, to laboratory glassware and equipment is well known [7] and contributes to poor recoveries.

This report describes the development and validation of a simple, specific and precise reversed phase liquid chromatographic method for the routine quality control of diminazene in the bulk drug substance and in pharmaceutical formulations. The major manufacturing impurity and degradant of diminazene aceturate have been resolved and identified by liquid chromatography-electrospray ionization mass spectrometry. The major impurity has been synthesized and its structure confirmed by NMR spectroscopy.

2. Experimental

2.1. Materials and reagents

HPLC-grade methanol, acetonitrile and analytical reagent grade formic acid (98% v/v), toluene, *n*-propanol and ammonia solution (35% v/v) were obtained from BDH Laboratory Supplies (Poole, UK). Ammonium formate (97%) and *p*-aminobenzamidine (98%) were purchased from Sigma-Aldrich chemicals (Dorset, UK). Diminazene aceturate (4,4′-diamidinodiazoaminobenzene diaceturate tetrahydrate) (99.6%) was a gift from Intervet, Germany and antipyrine (phenazone) (1,5-dimethyl-2-phenyl-pyrazolidin-3-one) (> 99%) was a gift from the Medicines Testing Laboratory (Edinburgh, UK). Preparative thin layer chromatography plates (PK6F silicaGel60, 1000 μm thickness) were purchased from Whatman Lab. Division (Clifton, USA).

2.2. Liquid chromatography

Analysis of diminazene was performed with an HPLC system consisting of a Spectra-SYSTEM P2000 pump (Thermoseparations) equipped with an online autosampler Spectra SYSTEM AS1000 (Thermoseparations) and 20 μl fixed injection loop. Analytes were monitored at 254 nm with a Spectra System 6000LP-photodiode-array detector (Thermoseparations). CHROMQUEST chromatography workstation software (Version 2.51, 1999) was used for instrument control and data acquisition.

Chromatographic separations were performed at ambient temperature on a Lichrospher® 60 RP-select B (C8) column(125 × 4 mm i.d.), 5 μm particle size, (Hewlett Packard, Germany,) with a 20 × 2 mm i.d. guard column (packed with Lichrosorb C8, 5 μm particle size; E. Merck, Darmstadt, Germany). The mobile phase (acetonitrile–methanol–ammonium formate buffer) (20 mM, pH 4.0) (10:10:80 v/v/v) was delivered at a flow rate of 0.7 ml/min. Ammonium formate buffer (20 mM, pH 4) was prepared by dissolving

1.2612 g ammonium formate in a 1000-ml volumetric flask with distilled water. The pH of the resulting solution was adjusted to 4.0 with formic acid and made up to the mark with distilled water.

2.3. Preparation of standard solutions

Exactly 50 mg of diminazene aceturate and antipyrine were accurately weighed, and transferred together into a 100-ml volumetric flask which was made up to the mark with water (500 μg/ml stock solution). The solution was then serially diluted with water to obtain standard calibration solutions at concentrations of 20, 40, 60, 80 and 100 μg/ml of diminazene aceturate and antipyrine.

2.4. Preparation of generic formulation products of diminazene

For the complete studies, generic samples (102 samples), consisting of about 17 brands of diminazene formulations were sourced from pharmacies and veterinary outlets in eleven countries in trypanosomosis endemic areas of Africa. Solutions of five of these generic samples of diminazene (stated content of diminazene aceturate and antipyrine was 0.555 and 0.625 mg/g of granules, respectively) were prepared by dissolving an accurately weighed amount (112 mg) of the granules in a 100-ml volumetric flask, which was made up to the mark with water. These solutions were then diluted to nominal concentrations of 50 and 62.6 μg/ml of diminazene aceturate and antipyrine, respectively, and analyzed by HPLC.

2.5. Method validation

2.5.1. Stability of solutions of diminazene and antipyrine

In order to demonstrate the stability of solutions of diminazene and antipyrine during analysis, a standard solution consisting of a mixture of diminazene aceturate (50 μg/ml) and antpyrine (50 μg/ml) was prepared in water and aliquots analyzed at specific time intervals: 2, 4, 6, 8, 24 and 48 h at room temperature (22 °C).

2.5.2. Specificity and robustness

The specificity of the method was confirmed by comparing the peak purity index values of chromatographic peaks of diminazene and antipyrine acquired from injections of standard solutions with those obtained from injections of generic samples. Standard solutions of diminazene and antipyrine were also spiked with solutions of the degradation product and the synthesized impurity of diminazene and the peak purity index values compared. This was to ensure that the analyte peaks were homogenous and that there was no interference from either degradation products or impurities of diminazene and any components of excipients. The peak purity index was determined by using the online CHROMQUEST workstation software. Robustness was assessed by determining the retention times of diminazene and antipyrine when two slightly modified mobile phases were used.

2.5.3. Precision and linearity of method

Precision of the method was determined by analysis of five replicates ($n = 5$) of standard solution mixtures of diminazene and antipyrine at three concentrations: 20, 50 and 100 μg/ml. To determine intra-day precision of the method, replicates ($n = 5$) of the three standard solutions were analyzed and for inter-day precision, replicates ($n = 5$) of freshly prepared standard solutions were analysed on three different days. The relative standard deviations (RSDs) of the analyte peak areas were determined.

The linearity of detector response for diminazene and antipyrine was determined by evaluation of regression statistics of five calibration curves acquired from replicate ($n = 3$) analysis of five different sets of calibration solutions of analytes prepared at concentrations of 20, 40, 60, 80 and 100 μg/ml.

2.5.4. Limits of detection and quantification

The limit of detection was established by determining the concentration of a dilute solution of diminazene that gave a signal to noise ratio of 3:1, while the limit of quantification was determined as the lowest concentration of diminazene

which gives an imprecision value of not more than 3% (RSD) for triplicate ($n = 3$) injections.

2.6. Identification of related substances of diminazene

2.6.1. Electrospray ionization mass spectrometry

Mass spectral identification of diminazene and its related substances (Fig. 1) was carried out with a Thermoquest Automass LC/MS instrument equipped with an electrospray ionization (ESI) interface and a quadrupole mass analyzer. The mobile phase consisted of a mixture of acetonitrile–methanol–ammonium formate buffer (20 mM, pH 4.0), 10:10:80 v/v/v, which was delivered at 0.7 ml/min, and nebulized by nitrogen gas at 480 °C into an electrospray mass analyzer operated in the positive ion mode with a cone voltage of 18 V and corona needle voltage at 2180 V. THERMOQUEST X-calibur software, was used for data acquisition.

2.6.2. Synthesis

2.6.2.1. Equipment. IR spectra were obtained with a MATTSON 5000 FTIR spectrophotometer and ^{1}H and ^{13}C-NMR spectra with a Bruker AMX 400 spectrometer.

2.6.2.2. 1-(4 Amidino phenyl)3-(4 carbamoyl phenyl)-triazene. The major manufacturing impurity of diminazene, 1-(4 amidino phenyl)3-(4 carbamoyl phenyl)-triazene was synthesized by refluxing a mixture of diminazene diaceturate tetrahydrate (300 mg) and anhydrous sodium carbonate (50 mg) in 50 ml of a mixture of methanol/water 1:1 (v/v) for 2 h over a steam water bath. The product (impurity) was isolated by preparative thin layer chromatography (mobile phase: *n*-propanol–toluene–conc. ammonia solution, 3:1:1 v/v/v) (Diminazene R_f 0.0; Product R_f 0.31). The silica in the region of the product in the chromatogram (R_f 0.31) was removed and extracted with methanol. The suspension was then centrifuged at $3500 \times g$ (10 min) to sediment the silica. The supernatant was removed and evaporated to dryness in a stream of nitrogen gas to yield the product. IR (KBr): $\upsilon = 3600$–2800 (NH), 1650 (C=O) cm^{-1}. ^{1}H-NMR (400 MHz, CD_3OD, δ Hz) 7.94 (2H, m, $J = 8.8$ Hz: H-3,5), 7.58 (2H brd, $J = 8.5$ Hz: H-2,6), 7.86 (2H, dt, $J = 8.9$ Hz: H-3′,5′), 7.63 (2H, brd, $J = 8.6$ Hz: H-2′,6′); ^{13}C-NMR (100 MHz, CD_3OD, δ ppm-exact assignment from HMBC spectrum) 171.1 (C=O, C-7), 167.9 (C=NH, C-7′), 151.6 (C–NH–N, C-1′), 150.8 (C–N=N, C-1), 132.3 (C-4), 124.5 (C-4′), 130.3 (C-3, 5), 130.8 (C-3′,5′), 117.9 (C-2′, 6′), 119.5 (C-2,6).

LC/+ESI-MS: m/z 283 $[M+H]^+$ base peak (Fig. 4d), m/z 255 $[M+H-N_2]+$, m/z 324 $[M+H+CH_3CN]^+$ acetonitrile adduct.

3. Results and discussion

Successful resolution of diminazene, antipyrine (*excipient*) and its related substances (Figs. 2 and 3A) was achieved with a base deactivated stationary phase column, Lichrospher®60 RP (C8) select-B. The presence of 'free silanol' groups in conventional silica based reversed phase columns, which occurs mainly as a result of incomplete end-capping, can give rise to residual interactions

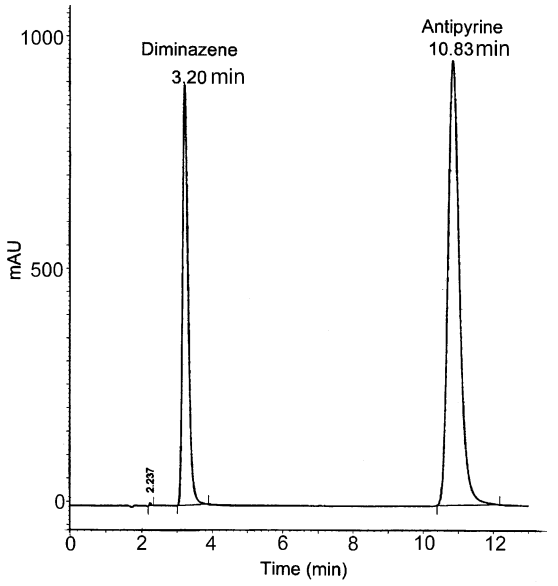


Fig. 2. HPLC chromatogram of diminazene and antipyrine monitored at 254 nm. Mobile phase: acetonitrile/methanol/HCO_2NH_4 (pH 4, 20 mM) (10:10:80 v/v/v).

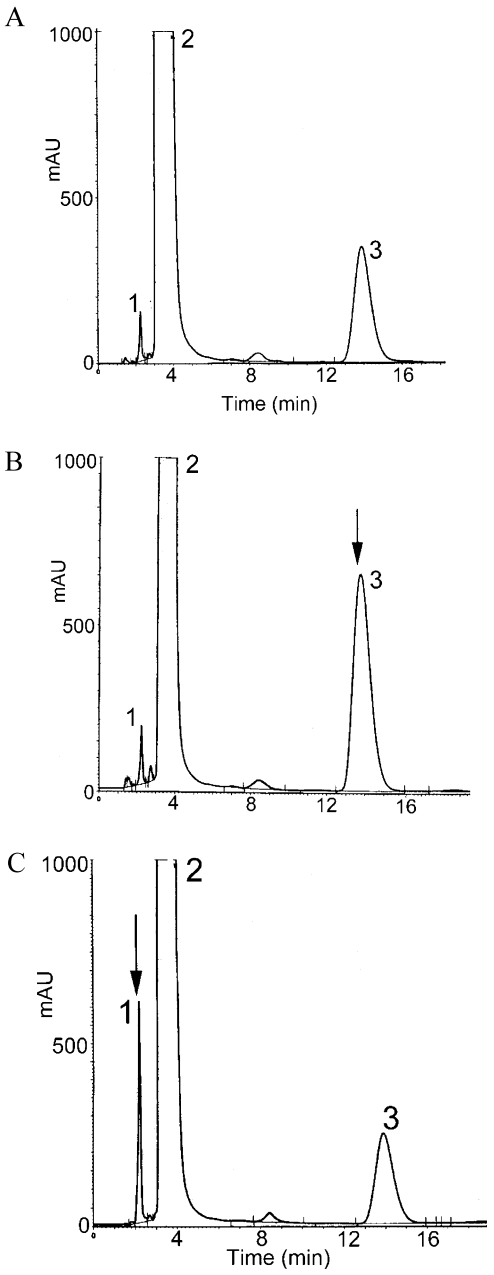


Fig. 3. (A) HPLC chromatogram of a solution (300 μg/ml) of diminazene aceturate showing a peak due to (1) a degradant, (2) diminazene, and (3) a manufacturing impurity. Chromatograms (B and C) were obtained after spiking a solution of diminazene with the synthesized impurity and standard degradant, respectively.

with highly basic analytes in chromatographic separations. This results in tailed peaks, which can dramatically impair quantification. However, the process of silica pretreatment (base deactivation) reduces the number of active silanols in order to produce relatively sharper peaks for basic analytes. Among the conventional reversed phase columns evaluated, the base deactivated column Lichrospher®-60 RP-C8 (5 μm particle size) gave a good peak shape for diminazene aceturate and antipyrine (Fig. 2) (Asymmetry factor at one-tenth peak height ≅ 1.10 for diminazene). The pH of the mobile phase was maintained at 4.0 in order to further suppress silanol ionization and reduce residual interactions with the basic amidino moieties of diminazene. The presence of ammonium formate buffer in the mobile phase provided competing ammonium ions that helped to improve the peak shape of diminazene.

3.1. Stability indication of assay

The stability of diminazene and antipyrine in solution during analysis was determined in order to evaluate the stability indicating capability of the assay. The results (Table 1) indicate that diminazene is stable during analysis and degradation in solution is only significant after 24 h storage at room temperature (22 °C). Cleavage of the triazene bridge of diminazene in acidic pH readily occurs and this may be due to an attack on a triazene nitrogen by hydronium ions (H_3O^+)

Table 1
Stability of diminazene and antipyrine in solution.

Time (h)	Diminazene, 50 μg/ml ($n = 3$)[a]	Antipyrine, 50 μg/ml ($n = 3$)[a]
0	100	100
2	99.2 ± 0.44	99.98 ± 0.03
4	100.1 ± 0.58	99.98 ± 0.16
6	98.29 ± 0.52	100.04 ± 0.13
8	97.98 ± 0.65	100.13 ± 0.22
24	94.55 ± 0.60	100.10 ± 0.25
48	91.28 ± 1.65	99.79 ± 0.12

[a] Values are % of initial concentration (mean ± S.D.) (100% at $t = 0$).

producing *p*-amino benzamidine as the major degradation product (Fig. 1).

3.2. Specificity and robustness

The peak purity index values of diminazene and antipyrine peaks in chromatograms of all the standard and generic sample solutions were above 0.997 indicating that there was no interference from impurities, degradation products or excipients and providing evidence of the selectivity of the proposed HPLC method. The chromatographic peaks of the degradation product and impurity (Fig. 3A–C) are clearly resolved from diminazene. Small changes in the mobile phase composition did not produce any major changes in retention times of either diminazene and antipyrine or the impurity and degradant, demonstrating the robustness of the method.

3.3. Linearity and precision

There was a rectilinear relationship between detector response and concentration over a concentration range of 0–100 μg/ml for diminazene aceturate and antipyrine (Table 2) which encompassed a nominal sample assay value for both analytes. This ensures that determination of analytes in formulation products can be performed using a single reference standard. Values for the imprecision of the assay, calculated as RSD of five replicate measurements were within acceptable limits (Table 3) for intra-day and inter-day assay variability. This demonstrated the ruggedness of the method, and its suitability for use in routine analysis (quality control) of diminazene aceturate in the bulk substance as well as in generic formulations.

Table 3
Intra- and inter-day assay imprecision for diminazene aceturate

Concentration (μg/ml)	Imprecision (% RSD)	
	Intra-day	Inter-day
20	1.6	2.3
50	0.4	1.1
100	0.2	1.2

RSD, relative standard deviation.

3.4. Limits of detection and quantitation

The limit of detection of diminazene (based on three times the average noise level) was 50 ng/ml, while the limit of quantitation was 10 μg/ml (RSD $< 3\%$, $n = 3$ injections).

3.5. Application of the HPLC method

The proposed HPLC method has been used in the analysis of more than 100 generic formulation products of diminazene and the results of five samples are shown in Table 4.

Within a tolerance window of $\pm 10\%$ of label claim of diminazene content (i.e. 90–110%), 24%

Table 4
Results of analysis of generic formulations (granules) of diminazene aceturate

Formulation	% of stated content*	
	Diminazene aceturate	Antipyrine
A	99.4 ± 0.82	95.8 ± 0.04
B	109.3 ± 0.12	86.8 ± 0.04
C	94.4 ± 0.22	99.7 ± 0.05
D	91.0 ± 0.13	99.8 ± 0.01
E	86.0 ± 0.07	103.2 ± 0.01

* Values are mean ± S.D. ($n = 3$) percentage of label claim.

Table 2
Regression statistics for diminazene and antipyrine

Analyte	Range (μg/ml)	*n*	Slope	Intercept	Correlation coefficient (r^2)
Diminazene	0–100	5	196 025 ± 986	454 924 ± 88 128	0.9996 ± 0.001
Antipyrine	0–100	5	439 045 ± 1482	317 556 ± 41 565	0.9999 ± 0.001

Slope, Intercept: mean ± S.E.; Correlation coefficient (r^2): mean ± S.D.

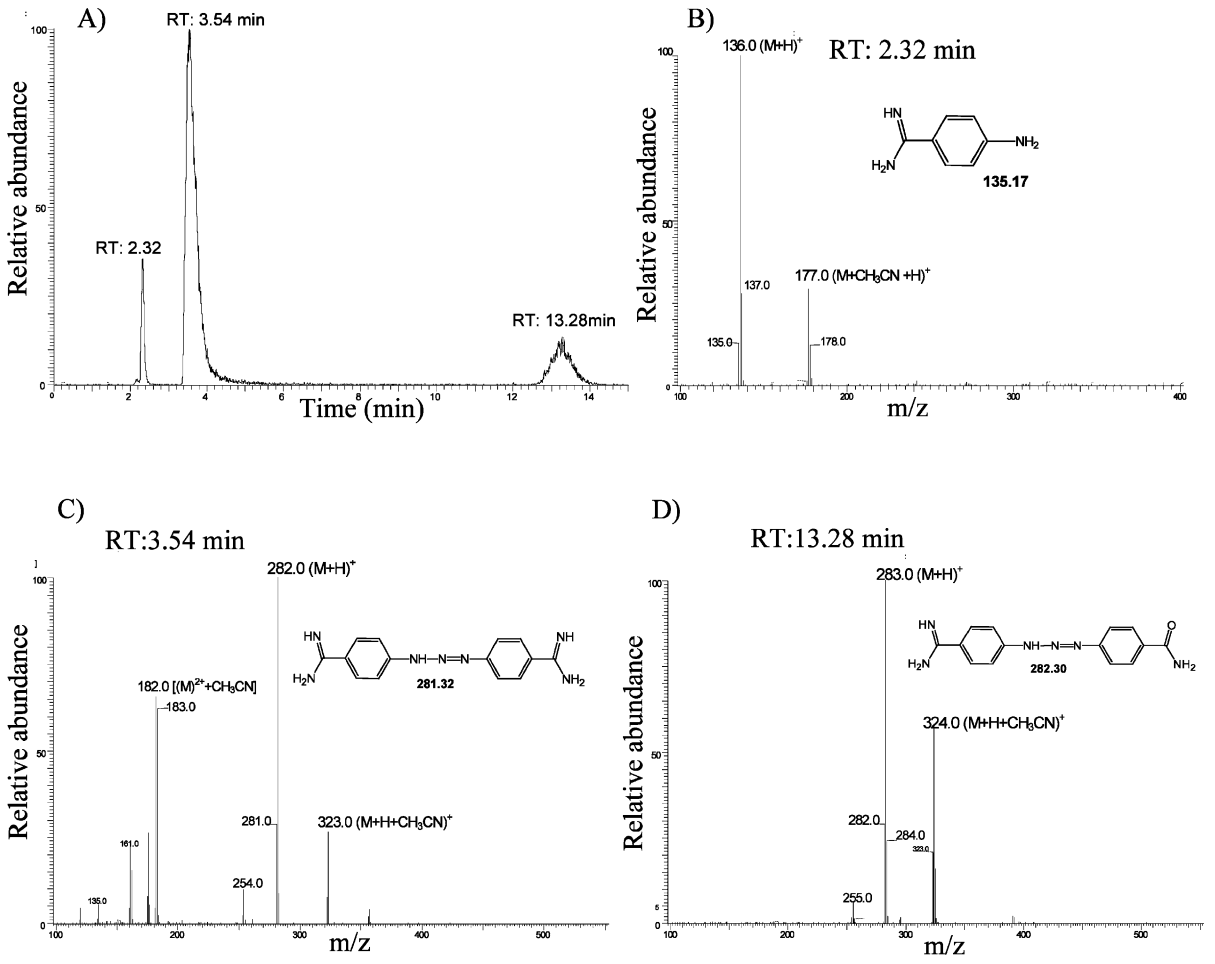


Fig. 4. (A) Selected ion mass chromatogram *m/z* 136, 182, 283 of a solution of diminazene monitored and product ion spectra of (B) degradant, (C) diminazene and (D) impurity peak.

of all the samples analyzed failed ($<90\%$ label claim), while 8% were found to be above the maximum limit ($>110\%$ label claim). The results gave an indication that the quality of generic products of diminazene aceturate in international commerce appear to be compromised which could contribute to drug resistance.

3.6. Related substances

ESI is a soft ionisation technique providing a sensitive means of analysis of a wide range of polar molecules. However, ESI generates multiply charged ions which results in the formation of adducts with the components of the mobile phase. The selected ion chromatogram of a concentrated solution of diminazene (300 μg/ml) (Fig. 4A), has three ion peaks monitored at *m/z* 136, 282 and 283 due to quasi molecular ions $[M+H]^+$ of a degradant (RT:2.32 min), diminazene (RT:3.54 min) and an impurity (RT:13.28 min), respectively. The product ion spectrum of the degradant (Fig. 4B) shows peaks at *m/z* 177 and *m/z* 136 due to the formation of an acetonitrile adduct ion ($[M+H+CH_3CN]^+$ and a protonated molecular ion $[M+H]^+$, respectively. The product ion spectra of diminazene and its impurity (Fig. 4C and D) show quasi-molecular ions (base peak) at *m/z* 282 and

283 for diminazene and its major impurity, respectively. The distinctive ions at m/z 182 and 183 in the ion spectrum of diminazene (Fig. 4C) are due to the formation of acetonitrile adducts of the doubly charged ions of diminazene $[(M)+CH_3CN]^+$ and $[(M)^{2+}+CH_3CN]$, respectively. The absence of ions at m/z 184, in the ion spectrum of the impurity is an indication of the absence of doubly charged ions and is consistent with the structure of the impurity being 1-(4 amidino phenyl) 3-(4 carbamoyl phenyl)-triazene.

As a further confirmation of the structure, the synthesized impurity (II), co-eluted with the manufacturing impurity present in generic formulations of diminazene (Fig. 3A and B). Similarly, the standard degradant (III) (p-amino benzamidine), coeluted with the degradation peak in the generic product (Fig. 3A and C). The UV spectra of the synthesized impurity (UV spectrum: water—$\lambda_{max} = 370$ nm) and the standard degradant (UV spectrum: water—$\lambda_{max} = 297$ nm) were matched with their respective peaks in the generic products of diminazene.

4. Conclusion

The developed method is specific, precise and accurate for the determination of diminazene aceturate in the presence of its related substances and excipient (antipyrine). It has been successfully employed for the quality evaluation of generic formulations of diminazene aceturate obtained from over ten trypanosomosis endemic countries of Africa. It is anticipated that this method will help to uncover potential substandard and counterfeit products of diminazene in international commerce. The major manufacturing impurity [1-(4 amidino phenyl)3-(4 carbamoyl phenyl)-triazene] and degradant (p-aminobenzamidine) of diminazene have been resolved and identified by ESI mass spectrometry.

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