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# Identification of the specified impurities of silver sulfadiazine using a screening of degradation products in different stress physico-chemical media

Bogdan I. Cioroiu [a], Mihai I. Lazar [a], Miguel A. Bello-López [b], Rut Fernandez-Torres [b,c,*]

[a] Department of Drug Analysis, University of Medicine and Pharmacy "Gr.T. Popa" Iasi, Universitatii Street No. 16, Iasi, Romania
[b] Department of Analytical Chemistry, University of Seville, Seville, Spain
[c] Research Centre of Health and Environment (CYSMA), University of Huelva, Spain

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ABSTRACT

Determination of silver sulfadiazine degradation products in several stress media was carried out by high pressure liquid chromatography (HPLC) with diode array detector (DAD) and hybrid mass spectrometer triple quadrupole-linear trap. The optimal chromatographic method used a Hypercarb column with a stationary phase 100% carbon, a mobile phase composed by a mixture 45:55 formic acid 1% solution and acetonitrile and detection at 275 nm. Structure elucidation was carried out on the mass spectrometry system using same chromatographic conditions and based on MS/MS techniques. Under these conditions up to 9 possible impurities were demonstrated to be degradation products respecting silver sulfadiazine evolution under different stress conditions: temperature, acid, basic, oxidation, reduction and catalyzed photodegradation. Sulfacetamide, sulfanilic acid (4-aminobenzenesulfonic acid), aniline, pyrimidin-2-amine, 4-aminobenzenesulfonamide, 4-methylidenesulfanilaniline, 4-aminophenol, 4-amino-n-methyl benzenesulfonamide and benzenesulfonic acid were identified by mass spectrometry in order to cover the possible degradation paths of silver sulfadiazine. Kinetics were also evaluated to obtain the prediction of shelf life of the substance. The linearity domain for the method was between 0.0005 mg/ml and 0.25 mg/ml for each compound. Recovery factors in accuracy determination were between 95 and 105% relative to target concentrations of silver sulfadiazine and the quantitation limit was 0.00025 mg/ml.



## 1. Introduction

Silver sulfadiazine is a sulfonamide compound used for the treatment and prevention of infections caused by severe burns and other skin disorders such as leg ulcers and for prophylaxis of infection in skin tattooing. Silver sulfadiazine, unlike sulfadiazine has a broader antimicrobial activity against gram positive, gram negative bacteria and some yeast, because of the fact that silver salts have a higher influence on the cell membrane and cell wall [1]. Under these conditions, there was reported a quite small systemic absorption, only a concentration of 10–20 micrograms/ml being achieved. The quantity could rise if the treated area is increased and the time of application is prolonged [2].

Noda et al. [3] showed that the type of excipients used in pharmaceutical formulations and the conditions of formulation are essential for the therapeutic efficiency. Also, can predict possible reactions which employs nucleophilic interactions with electrophilic excipients or for interactions between electrophilic substances (carboxylic acid derivates, esters, amides, halogens) and nucleophilic excipients.

According to the ICH Q3A guideline [4], different type of media can be applied in order to synthesize and simulate the rate of the degradation of active pharmaceutical substance such as basic and acidic, oxidation and reduction media, photo-degradation or temperature [5].

In the most topical formulations, silver sulfadiazine is dispersed, but the presence of different substances with specific character produce the redistribution of silver ions, so the problem of the study of the behavior in different stress media is reduced to the monitorization of the sulfadiazine.

Recent scientific studies on sulfadiazine and sulfonamides are concerned on the determination of the active pharmaceutical ingredients (APIs) and degradation products from watersamples [6–11], elimination from soil [12], food [13] and biological samples [14]. For better elimination of sulfonamides derivates from different matrixes it was concluded that there is a special need to involve some catalyzers for speeding up the reactions.

Presence of metal ions could create the conditions of acceleration of the instability of a certain substance based exactly on the

* Correspondence to: Departamento de Química Analítica, Universidad de Sevilla, C/Profesor García González s/n, 41012 Sevilla, Spain. Tel.: +34 9 545 564 42; fax: +34 9 545 571 68.
*E-mail address:* rutft@us.es (R. Fernandez-Torres).

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catalization properties. Metal salts as $FeCl_3$, $FeNO_3$were used for the elimination of some sulfonamides derivates from wastewater; the concentration of the ions was about just 10 mM and the reaction was performed on exposure to ultraviolet irradiation. Also in these cases compounds like titanium dioxide were used for complementary of reaction [15]. Using titaniumas excipient should be with high concern on the photo instable drugs based on its properties to produce hydrolysis reactions, similar with basic or acidic media [16]. Temperature and temperature/humidity are used in stability studies of pharmaceutical sunder accelerated conditions (e.g. 40 °C and 80%) for shelf life prediction of the active pharmaceutical ingredient in formulated products.

In all these previously mentioned studies there were used hyphenated techniques such as high performance liquid chromatography coupled with triple quadrupole or high resolution mass spectrometry in order to determine the concentration of the analytes and identification of the degradation products. Most of the methods were performed on reversed phase chromatographic columns using as mobile phase mixtures of acetonitrile and solutions of formic acid, an organic pH modifier compatible with mass spectrometry technology. Some degradation products such as sulfanilic acid, aniline, sulfacetamide, benzenesulfonamide, benzenesulfonic acid were identified [16].

The scope of the present study was to identify the impurities and degradation products of silver sulfadiazine under advanced stress conditions in order to identify the degradation products and their structure elucidation with an ion trap mass spectrometer, as well as establishing the best selectivity of the method by experimental design methodology using a high efficient chromatographic column suitable for conventional high performance liquid chromatography but also for ultrahigh pressure chromatography.

## 2. Experimental

### 2.1. Materials and reagents

All chemicals were of analytical-reagent grade or better. All solutions and dilutions were prepared with ultrapure water from a Milli-Q Plus water purification system (Millipore, Billerica, MA, USA). Acetonitrile-HPLC grade, formic acid, triethylamine, sodium acetate, nitric acid (65% purity), hydrochloride acid (37% purity) and sodium hydroxide, hydrogen peroxide (35%), ammonia (35%), formaldehyde (37%) and iron (III) nitratedecahydratewere supplied by Merck KGaA, (Germany). Silver sulfadiazine (SSD) was obtained from BioMolekula(Germany). Methyl p-hidroxibenzoate 99.3% (NPG) and propyl p-hydroxybenzoate 99.5% (NPS) were supplied by Sigma-Aldich Chimica (Romania).

Standard 5 mg $mL^{-1}$ stock solutions of SSD used for forced degradation studies were prepared by dissolving 50 mg SSD to 10 mL of each degradation media. Solutions of 0.25 mg $mL^{-1}$and 1 μg $mL^{-1}$ prepared by adequate dilution of standard stock solution with mobile phase (formic acid 1%: acetonitrile 45:55 %v/v) were used for HPLC-DAD and HPLC–MS analyses, respectively. All solutions were filtered through nylon 0.45 μm filter previously to HPLC injection.

### 2.2. Equipment and experimental conditions.

Analytical profiling of the degradation products of SSD was performed on an Agilent 1100 series equipped with a photodiode array UV–vis (DAD) and fluorescence detectors in series mode. Temperature was maintained using column thermostat at 25 °C, isocratic elution was performed on a quaternary pump at 0.2 mL/min and 10 μL were injected using a manual Rheodyne injector. The method was applied on a 3 μm particle size Hypercarb™Thermo Fisher chromatographic column (100 mm length × 2.1 mm i.d.). Mobile phase consisted of a mixture of acetonitrile and 1% formic acid solution (55:45, v/v) that was filtered (0.45 μm) and degassed before use.

Mass spectrometry analyses were carried on a UPLC Agilent 1290 system coupled with a triple quadrupole linear ion trap hybridQtrap3200 MS system from ABSCIEX, USA. Same chromatographic conditions were used. The mass spectrometer operated using electrospray interface system with Turbo V™ source with TurboIonSpray® probe (300 °C) in positive polarity mode with a mass/charge ($m/z$) ratio in the range of 50–300 $m/z$. The curtain gas was set at 20 (relative units). Spray voltage (IS) was set at 5.5 kV. Three different types of scanning modes (EMS+, ER+ and EPI+) were considered for the elucidation of the intermediate products chemical structure considering the precursors and the characteristics of the product ions. For EPI+ experiments a declustering potential (DP) of 50 V and a collision energy (CE) of 35 V were applied.

### 2.3. Stress conditions assays.

Forced degradation studies were performed under stress conditions according to the ICH Topic Q1A (R2) Stability Testing of New Drug Substances and Products [17] and the limit of the degradation products were established according with the daily intake at maximum 1% without any information about toxicological testing [5]. The following assays were performed:

1. Acid–basic hydrolysis degradation study was performed using SSD solutions in nitric acid or sodium hydroxide media at a concentration interval ranged between 1 and 5 M. Obviously, the use of hydrochloric acid was not considered due to the low solubility of silver chloride salts. Solutions were neutralized with nitric acid or sodium hydroxide before chromatographic analysis.
2. Oxidation–reduction media assay was performed exposing SSD solutions to a reduction media obtained with formaldehyde of a concentration of 35% (v/v). Oxidation media was obtained with hydrogen peroxide with a concentration of 20% (v/v).
3. Temperature stability test were performed by exposing a SSD solution to a temperature interval between 40 °C and 100 °C. Additionally, the dry substance was exposed to a temperature variation between 40 °C and 140 °C to check if there are relevant differences on the stability of the molecule.
4. Temperature/humidity test monitorization was performed on dry substance and solution by considering 80% humidity and a temperature variation between 40 °C and 100 °C.
5. Ultraviolet (UV) and visible (vis) light exposure under room conditions (23 °C) test was performed on SSD solution. UV and vis catalyzed test were performed according to the ICH Q1B [18] guideline using a radiant exposure flow with a spectral range of 320 nm and an intensity of 200 W h/$m^2$.

Aqueous 5 mg $mL^{-1}$ SSD solutions were submitted to the different stress conditions test. A volume of 0.5 mL was considered for each sampling time and diluted to 10 mL with mobile phase. Monitorization was performed using a solution of 50 mg SSD dissolved in 1 mL ammonia (25%) and 9 mL of each degradation media except for acid hydrolysis degradation and catalyzed photodegradation studies where 50 mg SSD were dissolved in 1 mL nitric acid (1 M) and 9 mL of each degradation media. Every degradation study was monitored by a total time interval of 10 h. In every case, blank solutions were prepared according to the sample protocol previously described and injected.

**Table 1**
Central Composite Design matrix and corresponding CRF response.

| Block | A | B | F1 | F2 | Rs1 | Rs2 | Rs3 | CRF |
|---|---|---|---|---|---|---|---|---|
| 1 | 1.000 | 0.000 | 11.2 | 35 | 0.197 | 0.000 | 0.000 | −11.303 |
| 2 | 0.000 | −1.000 | 6.5 | 20 | 3.540 | 4.130 | 1.770 | 20.440 |
| 1 | 0.000 | 1.000 | 6.5 | 55 | 1.180 | 2.950 | 4.130 | 7.760 |
| 3 | 1.000 | −1.000 | 11.2 | 20 | 0.197 | 0.000 | 0.000 | −11.303 |
| 2 | −1.000 | 0.000 | 1.2 | 35 | 10.403 | 16.768 | 3.105 | 65.076 |
| 2 | 1.000 | 1.000 | 11.2 | 55 | 0.197 | 0.000 | 0.000 | −11.303 |
| 1 | −1.000 | −1.000 | 1.2 | 20 | 12.421 | 9.316 | 6.211 | 68.947 |
| 3 | −1.000 | 1.000 | 1.2 | 55 | 6.097 | 8.162 | 16.717 | 46.375 |
| 3 | 0.000 | 0.000 | 6.5 | 35 | 4.130 | 3.540 | 3.540 | 23.210 |

### 2.4. Validation parameters of the method.

According to European Pharmacopoeia, the method validation was performed using specificity, linearity, accuracy, repeatability, intermediate precision, system suitability, limits of detection and the limits of quantification. Method specificity was established for silver sulfadiazine (SSD) in relation with the impurities found in the forced degradation studies and also with two substances: methyl parabene (NPG) and propyl parabene (NPS), that play a role of preservatives in the final formulation. Linearity of the response was studied for a concentration interval between 0.00005 and 0.25 mg $mL^{-1}$. Linearity interval was performed from the limit of quantification to the maximum expected concentration of the drug [19]. For the correlation of impurities with the concentration of the active substance, accuracy was calculated according to ICH Q1A guideline [17] by comparing the relative response factor of the impurity against the main analyte concentration. Limit of quantitation (LOQ) and limit of detection (LOD) were established considering the signal to noise ratio of 10:1 and 3:1, respectively. Repeatability was tested for solutions of SSD with concentrations of 0.01 and 0.005 mg $mL^{-1}$. Intra-day, inter-day precision and stability of the solution were monitored for a SSD concentration of 0.005 mg $mL^{-1}$ and determined by performing the analysis in two days by two different analysts. The stability was verified for 12 h at room temperature and visible light exposure.

## 3. Results and discussion

### 3.1. Optimization of chromatographic conditions

Hypercarb™, porous graphitic carbon column (PGC) presents a great specificity for polar compounds; the mechanism of separation consisting in dispersive interactions between analyte and surface of separation and, additionally, charge induces interactions with the polarizable graphite [20]. A highly efficient method for silver sulfadiazine with octanol–water partition coefficient (Poct) of 1.3 and an acid dissociation constant of pKa6.5 [21] was developed to identify possible degradation products. Selectivity was demonstrated in relation with the methyl parabene (NPG) and propyl parabene (NPS), compounds which are characteristic to the semi-topical formulations. Two pairs of peaks were identified; the resolution factor RS1 was established between SSD/NPG and the resolution factor RS2 was for NPG/NPS pair. The Box–Behenken experimental design was used for method development and consisted in monitoring the best resolution between the three compounds and the chromatography time. Other aspects like asymmetry or theoretical plates were not significantly affected so were not included in the study. Different mixtures of mobile phase constituents (formic acid (1%) and acetonitrile) (45:55 %v/v); (65:35 %v/v) and (80:20 %v/v) were used for the optimization of the retention time and selectivity. The pH profile was used to optimize the method parameters. A pH of 1.2, using the 1% formic solution, a pH of 6.5 of 25 mM solution of ammonium acetate and a pH of 11.2 (triethylamine) were employed. The experimental model was full factorial design and included 9 determinations, 2 independent factors (pH and acetonitrile content in mobile phase) and 1 experimental block. It was used a Chromatographic Response Function (CRF) in order to evaluate the quality of separation [18]. The data are presented in Table 1.

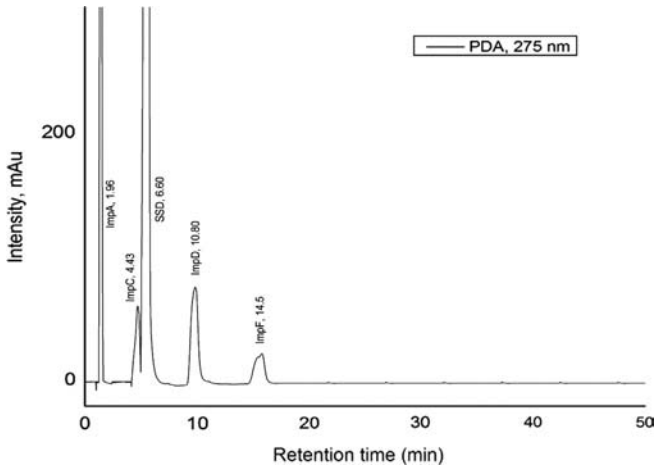


**Fig. 1.** Identification of the impurity *A* ($R_t$=1.96), impurity *C* ($R_t$=4.43), impurity *D*, ($R_t$=10.80) and impurity *F* ($R_t$=14.5) under UV catalyzed media.

The coefficients of the model were obtained by fitting the data using multilinear regression. The regression coefficients and their uncertainty at 95% confidence level were determined. The variance of the chromatography response function as a result of the variation of the independent factors along with their interaction is presented by the following equation:

$$CRF = 65.11 - 9.69pH + 0.20pH^2 + 0.83(\%ACN) - 0.015$$
$$(\%ACN)^2 - 0 \times pH(\%ACN)$$

From the relation it was concluded that the pH is the principal factor that affects the evolution of the parameters. The coefficient of correlation $R^2$=0.9805 and the coefficient of determination $R^2_{adjusted}$=0.9610 showed that the model of fitting is according to the Multilinear Regression. The chromatographic response factor, as a performance parameter is well predicted, confirmed by a coefficient of prediction ($Q^2$) of 0.868. The ANOVA analyze showed that the probability for the model regression is significant at 95% for pH ($p$=0.00002«0.05) and not significant for the %ACN variable ($p$=0.313 < 0.05). However, the model was not significant affected by the lack of fit because the predictive value was greater than 0.5.

Positive values in the linear regression parameters produced an increase effect of the CRF function, while the negative values showed a decrease of the response. Under these situations, the

**Table 2a**
Relative retention of impurities at different degradation media.

| | $t^0$ | Im*A* | Im*B* | Im*C* | SSD | Im*D* | Im*E* | NPG | Im*F* | Im*G* | Im*H* | Im*I* | NPS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Oxidation | 1.00 | 1.90 | 2.77 | 4.70 | 6.32 | 10.95 | 12.76 | 13.20 | 14.50 | 18.70 | 20.13 | 26.00 | 30.10 |
| Catalization | 1.20 | 2.20 | | 4.65 | 6.70 | 10.90 | 12.30 | – | 14.50 | 19.60 | 20.60 | 26.00 | – |
| Reduction | 1.30 | – | 3.33 | – | 7.80 | 10.35 | 13.70 | – | 15.00 | – | 25.00 | 32.10 | – |
| Acidic | 1.15 | 1.90 | 2.77 | 4.70 | 6.32 | 10.95 | 12.76 | – | – | 18.70 | 20.13 | – | – |
| Basic | 1.00 | 1.90 | 2.77 | 3.40 | 6.32 | 10.95 | 12.76 | – | – | 18.70 | 20.13 | 25.00 | – |
| Temperature | 1.20 | – | 2.20 | – | 6.70 | 10.90 | 12.30 | – | – | – | 20.13 | 26.00 | – |
| UV | 1.00 | 1.90 | 2.77 | 4.70 | 6.32 | 10.95 | 12.76 | – | – | 18.70 | 20.13 | – | – |
| Average retention time | 1.12 | 1.96 | 2.77 | 4.43 | 6.64 | 10.85 | 12.76 | 13.20 | 14.67 | 18.88 | 20.89 | 27.02 | 30.10 |

**Table 2b**
System suitability test data.

| | $t^0$ | Imp*A* | Imp*B* | Imp*C* | SSD | Imp*D* | Imp*E* | NPG | Imp*F* | Imp*G* | Imp*H* | Imp*I* | NPS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Peak width** | 0.10 | 0.40 | 0.50 | 0.60 | 0.60 | 0.60 | 0.60 | 0.60 | 1.00 | 1.00 | 1.30 | 1.50 | 1.60 |
| **Theoretical plates** | 0 | 533.02 | 681.19 | 1209.77 | 2717.45 | 7259.55 | 10044.9 | 10744.8 | 4775.47 | 7913.29 | 5734.37 | 7203.25 | 7856.81 |
| **Capacity factor** | 0 | 0.75 | 1.47 | 2.95 | 4.92 | 8.68 | 10.38 | 10.77 | 12.08 | 15.84 | 17.63 | 23.09 | 25.84 |
| **Selectivity factor** | 0 | 1.00 | 1.97 | 2.01 | 1.67 | 1.76 | 1.20 | 1.04 | 1.12 | 1.31 | 1.11 | 1.31 | 1.12 |
| **Resolution** | 0 | 0.00 | 5.39 | 5.84 | 6.28 | 10.28 | 4.51 | 1.02 | 2.03 | 5.61 | 2.04 | 5.24 | 2.45 |

final proportion of acetonitrile produced an increasing effect which is insignificant (+0.83) on CRF, whereas the pH produced an decrease of the function of −9.69. the interaction between the predictors produced no effect. The analysis was projected by surface response function build on the model at concomitant variation of the two factors. Fig. 1, The CRF was maximized for the following values of the parameters: the final concentration of acetonitrile (55%) and the pH of 1.2 which has the minimum value (codified with −1) (see Supplementary material S1,S2). The Pareto Chart of the standardized effects (Supplementary material S1) shows that with a confidence level of 95% just the pH independent value is producing a significant effect on the CRF. The obtained results (see Supplementary material S2) show the dependence between the desirability function and the factors of prediction.

Sulfanilic acid, anilinesulfacetamide, benzenesulfonamide and benzenesulfonic acid [22] were reported as relevant impurities of sulfadiazine [15]. Some of these substances present a strong absorbance at 275 nm [23–25]. Molecular spectrum obtained with silver-sulfadiazine at pH 1.2 shows two intense maxima at 240 and 278 nm. Usage of the 275 nm intense maxima assures the detection of the all the potential degradation products and also reveals a suitable selectivity regarding NPG and NPS. Additionally, the establishment of 275 nm wavelength, as discreet channel, assured the linearity of the signal for the concentration interval starting with 0.25 mg $mL^{-1}$ concentration up to the limits of detection and qualification.

### 3.2. Method validation

Method specificity was established for silver sulfadiazine in respect with the degradation products and also with NPG and NPS.

Relative retention time was calculated by dividing the retention time of the secondary compound between the retention time of an unretained substance ($t^0$) and reporting the retention time of the principal signal found on the solutions of analysis (Table 2a). Establishing the relative retention time [25] was suitable for the characterization of the specified impurities and assignation of the identified impurities.

The system suitability test included the evaluation of the efficiency or separation ($\alpha$), capacity factor ($k$), tailing factor ($T$), theoretical plates ($N$) and resolution ($R_s$). The results obtained are included in Table 2b. As it can be seen, all the signals have a good separation, only a resolution $<1.5$ is considered between NPG and impurity *E*.

Validation parameters were established according to Section 2.4. Linearity response was studied in the range of 0.0005–0.25 mg $mL^{-1}$ SSD. Correlation coefficient obtained was 0.9998, the maximum value of standardized residuals was of 1.87 and the Pearson coefficient value was of 0.94 which assured the good linearity and predictability of the regression model.

LOQ and LOD were established by the ratio signal-to-noise and also, using the statistical model which involves the standard error and the slope from linearity evaluation. Standard deviation of the intercept was found to be of 80.77 ($p>0.05$) and the slope value was of 224.019. Using the method calculation proposed by [25] in order to establish the correlation between linearity, limit of quantification and range, LOD for silver sulfadiazine was 0.001 mg $mL^{-1}$and LOQ was of 0.003 mg $mL^{-1}$. Using the chromatograms from blank samples and for the lowest concentrations used for the standard linearity test, there were found a S/N values of 3.21 and a S/N of 9.34 for 0.00005 and 0.00025 mg $mL^{-1}$, respectively that were according to the actual LOD and LOQ.

The repeatability was evaluated from SSD solutions of 0.005 mg $mL^{-1}$and 0.01 mg $mL^{-1}$ ($n=6$). Relative standard deviation values of 1.47 and 1.63 were obtained, respectively. The retention time deviations were of 0.89% and 1.78% respectively.

Intermediate precision was evaluated from the analysis of a 0.005 mg $mL^{-1}$ SSD solution in two consecutive days. The relative standard deviation were of 2.21% for peak area (mean value being of 1754 mAU) and 2.92% for retention time. Table 3 shows the accuracy from SSD solutions submitted to the different degradation media.

Accuracy and precision was determined for spiked samples at different concentrations in the most relevant degradation media. These samples were analyzed in a period of maximum 1 h from the preparation time. The recoveries were between 95% and 105% and the relative standard deviation between samples was of maximum 5%.

Table 3 shows the different validation parameters and the accuracy obtained in different stress media.

### 3.3. Degradation of silver sulfadiazine

The identification of degradation products under various stress conditions was investigated and the profiles of degradation are

**Table 3**
Validation parameters and method accuracy established by spiking known concentrations of sulfadiazine in different stress media.

| Parameter | Results | | | |
|---|---|---|---|---|
| | $X_{media}$ | SD | RSD% | |
| Inter-day precision (area) | 1773 | 28.3 | 1.60 | |
| Inter-day precision (retention time) | 6.77 | 0.05 | 0.73 | |
| Intra-day precision (area) | 1754 | 38.9 | 2.22 | |
| Intra-day precision (retention time) | 6.69 | 0.20 | 2.92 | |
| Stability of solution (12 h) | −0.6% | | | |
| Linearity | 0.9998 | | | |
| Interval (mg/ml) | 0.00005–0.25 | | | |
| | | **0.1 mg/ml** | **0.01 mg/ml** | **0.0025 mg/ml** |
| Recoveries | Oxidation media | 0.103 | 0.010 | 0.00241 |
| | | 0.101 | 0.010 | 0.00251 |
| | | 0.103 | 0.010 | 0.00245 |
| | Catalization/vis exposure | 0.103 | 0.010 | 0.00252 |
| | | 0.102 | 0.010 | 0.00260 |
| | | 0.101 | 0.010 | 0.00261 |
| | Acidic media | 0.099 | 0.010 | 0.00235 |
| | | 0.107 | 0.010 | 0.00248 |
| | | 0.098 | 0.010 | 0.00246 |

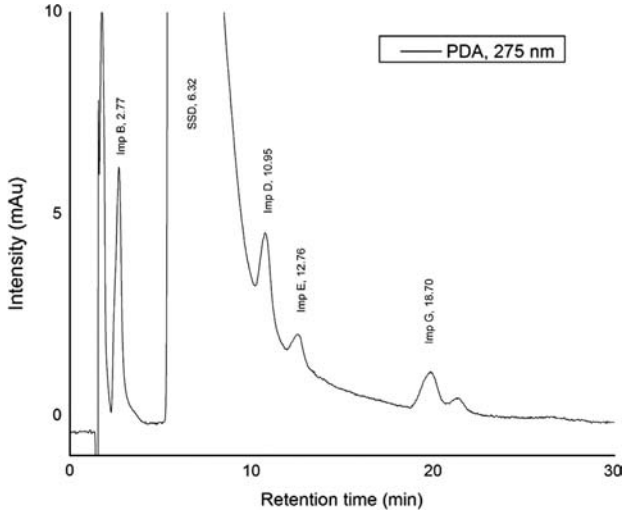


**Fig. 2.** Identification of the impurity *B* ($R_t$=2.77) impurity *D* ($R_t$=10.95), impurity *E* ($R_t$=12.76) and impurity *G*, ($R_t$=18.7) under acidic media.

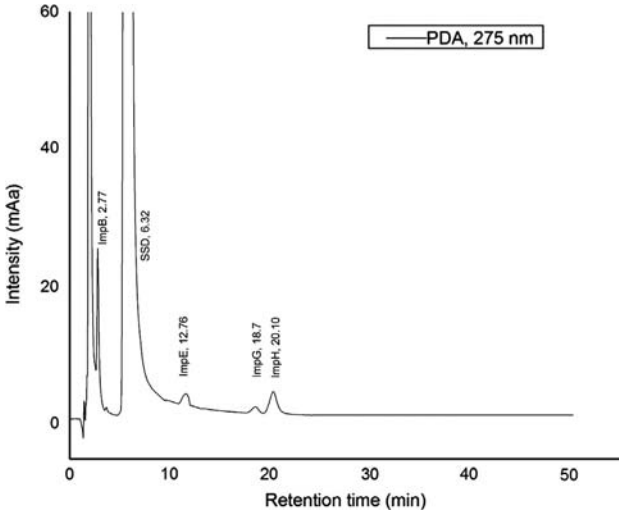


**Fig. 3.** Identification of the impurity *B* ($R_t$=2.77), impurity *E* ($R_t$=11.4), impurity *G*, ($R_t$=18.7) and impurity *H* ($R_t$=20.10) under oxidation media.

presented in the Figs. 1–4. Correlation between the decrease of signal of sulfadiazine and the related increase of the impurities found was realized by correlation matrices. In this sense, correlation Fisher coefficients were involved in the characterization of the impurities. For the correlation of impurities with SSD it was calculated the relative concentration by comparing the relative response factor of impurity detected versus SSD concentration. Table 4 shows the relative concentration of silver sulfadiazine impurities obtained at the final sampling step under the different degradation media.

### 3.3.1. *Acidic–basic hydrolysis.*

Firstly, 1 M nitric acid and 1 M sodium hydroxide solutions were used and no degradation was observed. The concentration of the media was progressively increased, until a final concentration of 5 M. A maximum monitorization time of 10 h was evaluated. Impurities *B* and *E* were revealed to be significant. Correlation coefficient on impurity B was of −0.94 and the increase of the content was up to 0.83% and for impurity *E* was of −0.84 with a percent of 0.34. In the study there were identified also impurity *D*

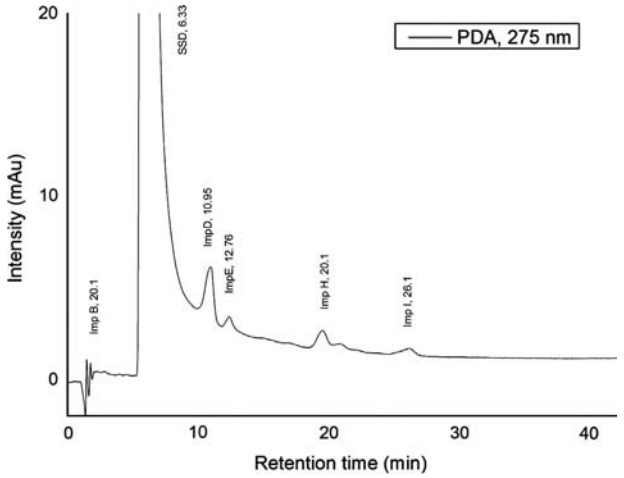


**Fig. 4.** Identification of the impurity *D* ($R_t$=10.95), impurity *E* ($R_t$=12.76), impurity *G*, ($R_t$=18.7) and impurity *I* ($R_t$=26.1) under temperature/humidity exposure.

**Table 4**
The degradation products profile obtained in different degradation media. The values are expressed as relative percentage concentrations.

| % concentration | Impurity | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Imp*A* **(0.295)**[a] | Imp*B* **(0.417)**[a] | Imp*C* **(0.667)**[a] | SSD **(1.000)**[a] | Imp*D* **(1.634)**[a] | Imp*E* **(1.922)**[a] | Imp*F* **(2.209)**[a] | Imp*G* **(2.843)**[a] | Imp*H* **(3.146)**[a] | Imp*I* **(4.069)**[a] |
| Solution/temperature | 0.013 | 0 | 0 | 99.86 | 0 | 0.024 | 0.000 | 0.101 | 0 | 0 |
| dry Substance/temperature | 0 | 0 | 0 | 99.84 | 0 | 0 | 0.037 | 0.117 | 0 | 0 |
| Dry substance /temperature/ humidity | 0.004 | 0 | 0 | 99.87 | 0 | 0 | 0.027 | 0 | 0 | 0.093 |
| Reduction | 0 | 0.230 | 0 | 97.39 | 0.362 | 0.192 | 0.433 | 0 | 0.950 | 0.440 |
| Oxidation | 0 | 6.070 | 0.469 | 92.93 | 0 | 0.087 | 0 | 0.105 | 0.330 | 0 |
| Vis-catalyzed | 0 | 10.082 | 0 | 83.39 | 0 | 6.247 | 0 | 0.000 | 0.282 | 0 |
| UV-catalyzed | 0 | 18.638 | 5.503 | 65.28 | 0 | 7.620 | 0 | 2.954 | 0 | 0 |
| UV exposure | 0.040 | 0.000 | 0 | 99.84 | 0 | 0.025 | 0 | 0.069 | 0.026 | 0 |
| Acidic media | 0 | 0.837 | 0 | 98.71 | 0.347 | 0.342 | 0 | 0 | 0.103 | 0 |
| Basic media | 0.019 | 0 | 0 | 99.89 | 0 | 0.030 | 0 | 0 | 0.060 | 0 |

[a] Relative retention time.

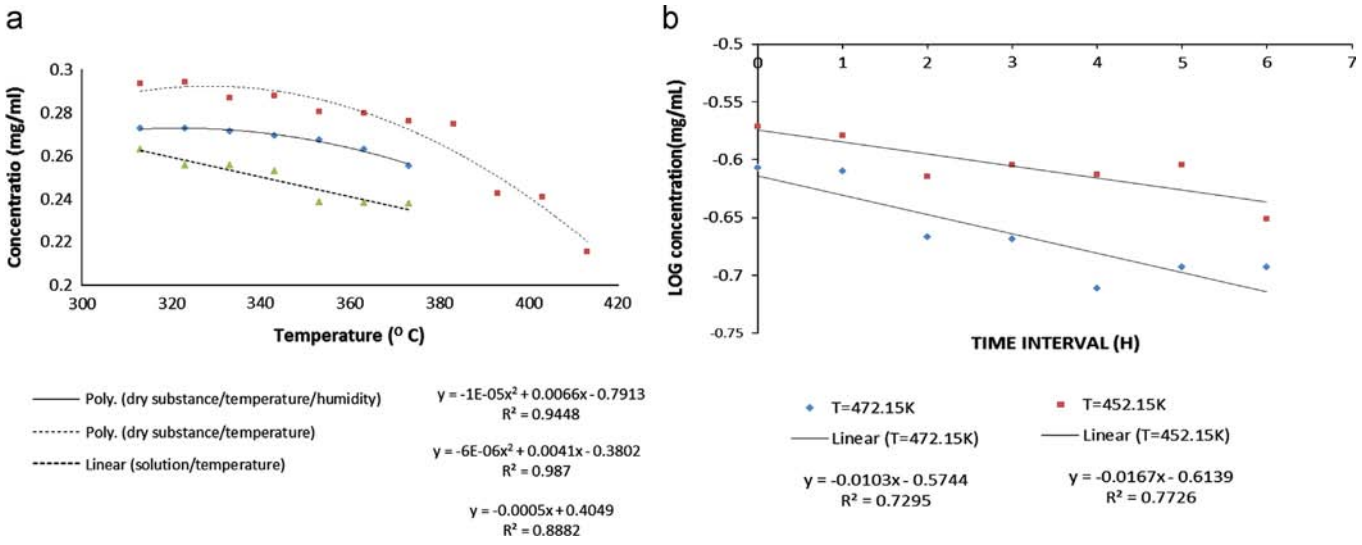


**Fig. 5.** (a) SSD degradation in relation with temperature variation. (b) Concentration variation during time variation (up to 6 h).

and impurity *G*, but the relative percentage concentrations had a lower level, maximum being of 0.1%. The presence of impurities and the decrease of the SSD concentration up to 98.7% confirm its instability in acidic media.

### 3.3.2. *Oxidation–reduction media.*

Oxidation was considered with respect to the perspectives of reactions with strong oxidant agents. Reactions with oxidizing agents were considered due to some constituents of pharmaceuticals could have the same influence mechanism. Reduction media, using formaldehyde, was considered by the fact of the change of oxidation state of the carbon atoms with concomitant producing carbon–carbon covalent bonds on the cleaved molecule of silver sulfadiazine exposed to the degradation media [26].

High instability of silver-sulfadiazine was observed when a 20% hydrogen peroxide solution was used as oxidizing media. During the monitorization interval, impurity *B* increased up to 6% relative concentration, impurity *C* had a value of 0.46% and the impurity *H* a concentration of 0.33%. The decrease of the concentration of silversulfadizine to 92% was co-related with previously mentioned impurities $R=-0.78$, $R=-0.77$ and $R=-0.74$ respectively. Impurity *I* was also considered because of the relative concentration of 0.44%, progressive increased during the monitorization.

Reduction media had a less active role on the degradation of silver sulfadiazine; formaldehyde concentration of 35% only provokes a decrease of the SSD concentration to 97.39% and the formation of impurity *B* (relative concentration of 0.23% and $R=-0.94$) and another impurity which was found as impurity *H* with a relative concentration of 0.95% ($R=-0.95$).

### 3.3.3. *Photo-degradation and photo-catalyzed degradation.*

Photo-degradation studies were performed at room temperature and humidity. Photo-degradation, performed under visible and in ultraviolet light, does not produce relevant SSD degradation, just a slight change of the color of the solution. Exposure to UV light, after 10 h, revealed the same concentration decrease of 99.66% and identification of the impurity *A* ($R=-0.93$), impurities *G* ($R=-0.8$) and *H* ($R=-0.73$) as potential degradation compounds because their final concentration was below 0.1%.

When an iron (III) salt was tested as possible catalyzer the photodegradation results changed dramatically. Exposing a solution of silver sulfadiazine solubilized in nitric acid 2.5% solution and 10 mM iron (III) at visible light produced a decrease of the concentration of silver sulfadiazine up to 83% and up to 65% if UV light was used. Impurity *A* raised up to 11.3% ($R=-0.96$) and impurity *D* up to 6.24% ($R=-0.93$). Exposing to UV beam (220 W/m$^2$) produced an

increase of the concentration for impurity *A* up to 18.63% ($R=-0.99$) and for impurity *D* up to 7.61% and correlation coefficient of $-0.91$. Impurity *C* was also identified from the time T3 (3 h) and rose up to 5.50% relative concentration ($R=-0.91$). Impurity *F* was confirmed as degradation product, potentially identified in exposure to UV-uncatalyzed, final concentration being 2.95% ($R=-0.99$).

### 3.3.4. Temperature/humidity

The effect of the temperature on the stability of SSD solution and solid substance, (40–100 °C range), was checked. Additionally the combination of a high relative humidity (80%) was tested on solid SSD between 40 and 140 °C.

All the impurities found on the previous degradation studies tested were present, but their concentrations were lower than the oxidation–reduction, acidic and basic hydrolysis and photo-degradation catalyzed, non-catalyzed assays. Good correlation were found respecting impurity *I* ($R=-0.93$), impurity *H* ($R=-0.80$) and impurity *B* ($R=-0.82$). Maximum relative concentration was of 0.11% for impurity *B* in respect with SSD.

### 3.4. Degradation kinetics of SSD under thermal conditions.

Temperature domain was established in order to verify the behavior of silver sulfadiazine and try to establish a pseudo-kinetic model of degradation dependent on temperature and also temperature/humidity. Based on signal amplitude and correlate with the concentration of the analyzed solution, a decrease of signal amplitude is obvious in every media of degradation. Correlation with other similar media (e.g. temperature and temperature/humidity for dry substance) showed that the highest rate of degradation of silver sulfadiazine is produced after 100 °C and the trend line was polynomial ($R^2=0.987$). The same trend line was suitable for characterizing the degradation process in case of temperature/humidity monitorization. Degradation for the solution showed a tendency suitable for a linear model, the correlation coefficient being $R^2=0.888$. In Fig. 5a there are concluded the variations for the 3 analysis systems.

The reaction constant, half-life period and energy activation were determined for SSD (0.25 mg $mL^{-1}$) solution at 80 °C and 100 °C for a time interval of 6 h, every sample being taken at 1 h time interval. Methods of calculation were considered after the Arrhenius kinetics conditions [27]. In these conditions the degradation rate constant determined by the slopes were found to be $8.17\times10^{-5}\,h^{-1}$ for 473.15 K and $5.269\times10^{-5}\,h^{-1}$ for 453.15 K respectively. The half-life periods ($t_{1/2}$) values were found to be about 8.482, 2 h at 472.15 K and 13.100 h at 452.15 K. In these conditions the activation energy was of 9.301 kcal/mol determined from the Arrhenius equation using absolute temperature values (453.15 K and 453.15 K) and the gas constant of 1.987 cal/mol (Fig. 5b).

## 4. Characterization

The identification of degradation products was carried by LC–MS triple-quadrupole hybrid linear trap with electrospray ionization. There were created the premises to produce the fragmentation path of the compounds using the positive ionization mode. For SSD, enhanced single ion scanning mode produced the isolation of the precursor ion with *m*/*z* 251.1 and the ion product scanning mode created the following product ions: *m*/*z* 251,233, 214.9, 205.9, 185.2, 170.9, 156, 152.9, 156, 134.9 108, 96, 92, 85, 65. All these ions correspond to different fragmentation sites of molecule along with different possible adducts which can be formed with acetonitrile, water or ammonia [28]. Modifications produced in ion product ionization scan mode are depicted in Fig. 6. Ions with *m*/*z* of 170 could correspond to the cleavage of the covalent bond between pyrimidinyl ring and amine group in position 4. Ions with *m*/*z* of 158 could correspond to the cleavage of 4-amino-pyrimidin-2yl group and benzenesulfonamide group and can be the fact of the cleavage of the covalent bond between aminobenzenic group and the sulfon-4-amine-pyrimidinic fragment. Also, another possible

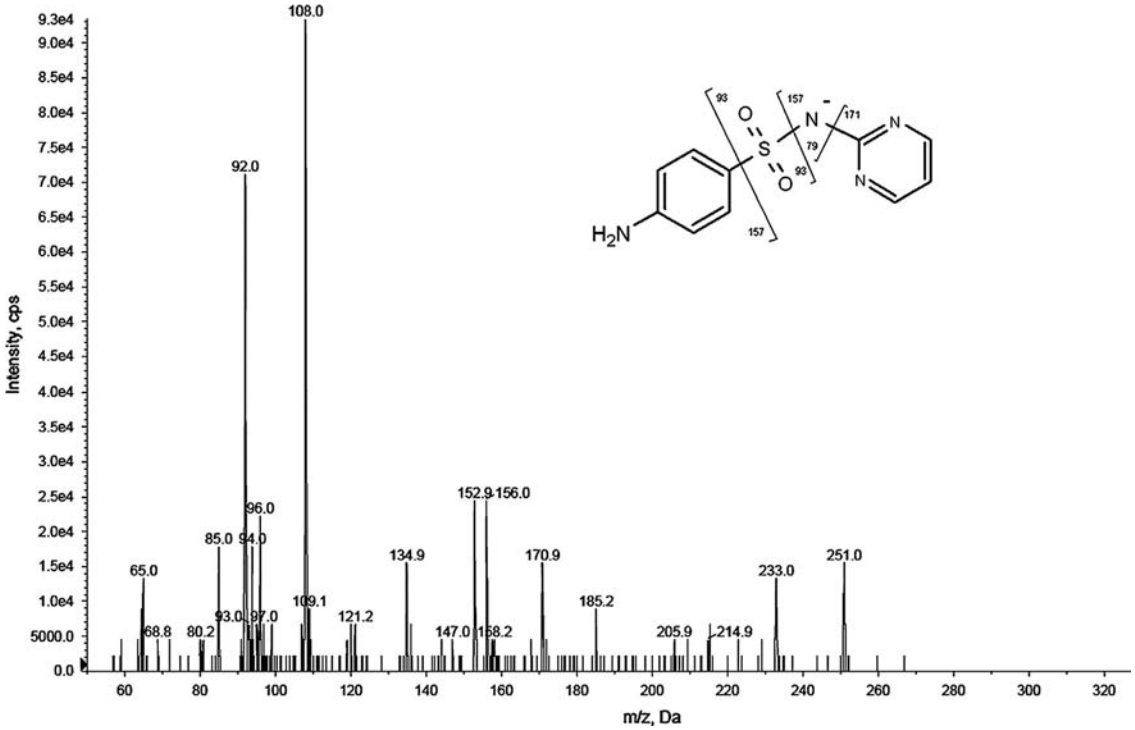


**Fig. 6.** Fragmentation paths of sulfadiazine. Possible cleavage paths and ion product mass spectra.

**Table 5**
Mass spectra characteristics of the identified impurities.

| Identified compound | Proposed structure | Precursor (ESI+) | Fragment products (EPI+) | Intensity |
|---|---|---|---|---|
| Impurity *A* | Sulfacetamide | 215.1 | 215.4; 108; 92 | 5.00 e+05 |
| Impurity *B* | 4-aminobenzene sulfonic acid | 173.0 | 170.2; 151.8; 134.9 93 | 5.00 e+05 |
| **Sulfadiazine** | **-** | **251.1** | **251; 233; 170.9; 156; 108; 92** | 9.30 e+04 |
| Impurity *C* | Aniline | 132.1 | 132; 131.97; 90.8 | 5.80 e+05 |
| Impurity *D* | Amino-pyridine | 137 | 139.1; 98.9; 92.9; 68.9 | 6.20 e+05 |
| Impurity *E* | 4-aminobenzensulfonamide | 173 | 173; 93; 90.8 | 6.40 e+05 |
| **Impurity *F*** | **For 3-cyanobut 3-enimidamide** | **134** | **136.1; 118.9; 109.1; 95.2; 76.8** | **3.80 e+05** |
| Impurity *G* | 4-aminophenol | 114 | 114.1; 98.9; 97; 80.9; 72.7 | 4.00 e+05 |
| Impurity *H* | Benzensulfonamide | 187 | 188.3; 159; 141; 132.2; 119; 97 | 5.00 e+05 |
| Impurity *I* | Benzenesulfonic acid | 158 | 158.2; 143; 115; 78.2; 60.7 | 5.00 e+05 |

path of degradation could produce in relation with the cleavage of the C4-N bond in the pyrdimidic group which further could be opened in the linear distribution.

### 4.1. Characterization of the degradation products

Analysis were performed in Enhaced Product Ionization (EPI) which permits the isolation of the precursor ion, trap it in the linear ion trap and further scan in order to produce corresponding characteristic fragments. Most of the degradation products were produced by the cleavage of the intern covalent bonds along with characteristic reactions produced by the specificity of the medium involved in the degradation process (e.g. acidic or basic hydrolysis, oxidation, catalysis). The mass scanning domain was established between 50 and 300 *m*/*z*, so charged ions with lower molecular mass could not be detected but they could be identified by their adducts. The results obtained are resumed in Table 5 and discussed below.

Impurity *A* is created in the oxidation and photodegradation media, in the presence of the iron. The sulfacetamide structure is determined by the electrophilic addition of the acetamide group to the sulfanyl aniline compound. Acetamide is produced by the retrosynthesis and cleavage of C1–N6 and C2–C3 in oxidative conditions [29]. Along with the retrosynthesis, the acetamide group is deprotonated in the basic media, which favors the electrohpilicadition with the sulfanyl aniline group. The general mechanism is proposed in Supplementary material S3a. Sulfacetamide was also confirmed in other studies [16].

Impurity *B* was considered as sulfanilic acid [16] in respect of the *m*/*z* ratio of 173 and the ion products with *m*/*z* of 173 and 93. In fact, *m*/*z* 93 corresponds to the compound, as a product resulted by the cleavage of the covalent bond between sulfonic radical and the adjacent amino in sulfadiazine molecule. Also, methyl-sulfonyl aniline could be confirmed with the same relative retention time (RRT=0.417) and *m*/*z* ratio of 171.1 in acidic conditions but with ion products with *m*/*z* 155, 134 and 97.1 ($\delta$=4.1 ppm).

Impurity *C*, a possible synthesis byproduct, was identified with a relative retention time (RRT=0.667) and the *m*/*z* ratio was of 132 for the ion precursor and 132 and 90.8 for products ions. Presence of the 132 ion product could be the result of a adduct formation with acetonitrile (*m*/*z* 132) and the fragment of 90.8 could be characteristic to aniline (effective *m*/*z* 93). The error was of about 2.2 ppm.

Impurity *D* appears at relative retention time with silver sulfadiazine of approx. 1.634 in the all the degradation media there was identified a signal which gave two possible structures. First it was identified the protonated ion with *m*/*z* ratio of 139.1 in acidic and reduction media with products ion of 139.1, 98.9, 92.9 and 68.9. Presence of 139.1 fragment showed the possible structure of amino-pyrimidine (*m*/*z* 93) identified by a possible adduct with acetonitrile (*m*/*z*139). Also, in a second alternative, using UV-degradation it was identified the protonated ion with *m*/*z* 137 and the fragmentation ions with *m*/*z* 118.8 and 92.9. The first product is considered a hydrate and the signal with 92.9 is related to the same compound 2-amino-pyrimidine. ($\delta$= −0.1 ppm).

Impurity *E* was characteristic to acidic, basic, temperature and UV degradation media. In all cases, the signal with a relative retention time of 1.922 gave a mass spectrum in ESI+ mode of 193.9 for acidic, basic and temperature and a protonated ion with *m*/*z* 171.1under UV degradation exposure. In all cases, the product ions had signals at 170, 153, 135, 105 *m*/*z* ratio. Difference between 193.9 molecular ions and 171.1 was associated with a possible adduct with sodium ions which was not formed in the UVphoto-degradation conditions. Presence of the product ion 93 confirmed the structure proposed: 4-aminobenzenesulfonamide. Other studies considered as a relative compound to be benzenesulfonamide [16].

Photochemically induced oxidation is conducted in the presence of UV light, a medium with high energy. In the presence of the UV media, an excitation of the drug molecules is produced and creates peroxides or epoxides with the oxygen from the media [30].

The presence of the principal signal in MS spectra with *m*/*z* 93.9 belongs to the amino-pyrimidil group. Ultraviolet irradiation produces intermolecular rearrangements for the aminopyrimidil groups [31]. Also, the intramolecular rearrangements are produced at the high temperatures, the molecular spectrum being identified in the temperature degradation media [32].

Impurity *F* relative retention time of 2.209 in the media which contain $Fe^{2+}$, according to the proposed mechanism in Supplementary material S3b is 3-cyanobut 3-enimidamide. This is based on the fact, that the amino-pyridimidineprodused an ring opening in the conditions of UV light, followed by the cleavage of the C4–C5 covalent bond and concomitant addition of resulting cyanopropil group to C3, based on increase density of protons determined by effect +I of amine groups. The mass spectrum has the following characteristics 136.1, 118.9, 109.1, 96, and 76.8. In these conditions, the product compound is an adduct with acetonitrile (41) and by removing this adduct, the compound has a mass of 95. By considering the *m*/*z* ratio of 96, the resulting compound is 3-cyanobut 3-enimidamide.

Impurity *G*, characteristic to catalysis, acidic and basic media had a effective relative retention time of 2.843 and the mass spectral characteristics 114 for protonated molecular ion but with 114.1, 98.9, 97, 80.9 and 72.7 *m*/*z* for product ions respectively. This induced the possibility of the presence of 4-aminophenol resulted under the assumptions that the compound is formed by the cleavage of the covalent bond between pyramidal and adjacent amino radical. Presence of hydroxyl group can be justified by the hydrolysis reaction produced in these media conditions.

Impurity *H* is a reaction product between sulfadiazine with the formaldehyde in basic media. First, between the ammonia and formaldehyde is produced an intermediate called formaldimine, which is extremely reactive and un-ionizable. Delocalization of the charges bases because of the difference of electronegativity

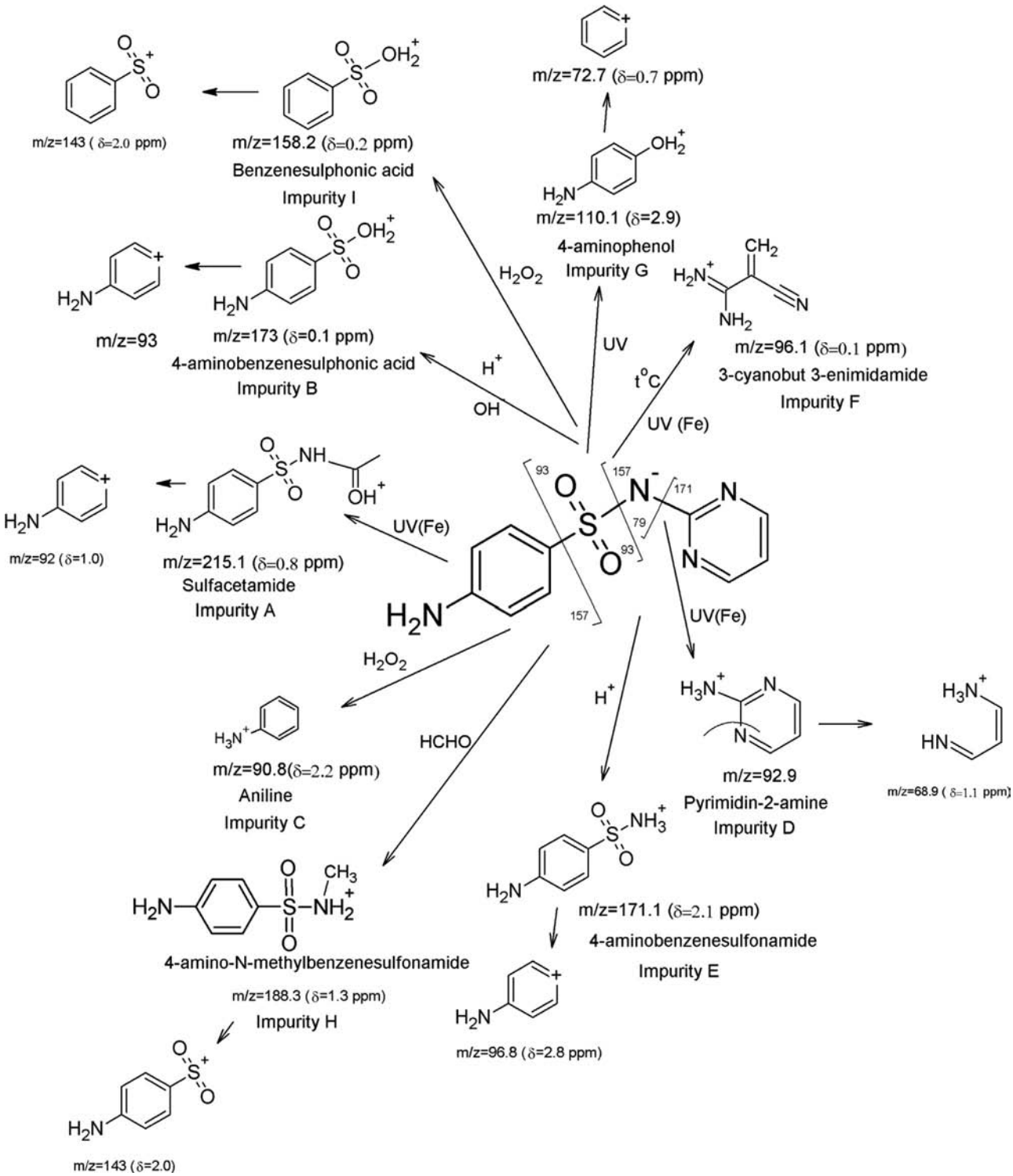


**Fig. 7.** Possible mechanisms for producing the degradation products.

between the nitrogen and carbon atoms produces the reactions with aromatic compounds.

The addition is nucleophilic based on the electrometric effect *E* present on the nitrogen atom. The reaction is produced according to the following equation [33]. The principal reaction is considered to be a nucleophilic addition between the formaldimine intermediate with a carbonyl group followed by the dehydration of the Schiff base. The reaction is produced on the primary and secondary amine group. Following the mechanism, the sulfon-aminophenol group produces an adduct rapidly with the intermediate (Supplementary material S3c). The supplementary spectral informations are given by the UV spectra provided by the DAD detector. Impurity *F* is considered a derivate of aniline. The molecular spectrum with a maxima at 290 nm is highly correlated with aniline spectrum, in contrast with molecular spectrum of pyrimidine which has the intense maxima at 208 nm and 232 nm.

The mass spectrometry determinations demonstrated a protonated ion of *m*/*z* 188.3 (relative retention time of 3.146), which is associated with the compound 4-amino-N-methylbenzenesulfoneamine. This is confirmed by the fragment ion of *m*/*z*-97

which is associated with amino-benzene which, in these conditions the carbon atoms lose their aromaticity because of the complete saturation, the ratio *m*/*z*-97 is more corresponding to the amino-ciclohexane.

Impurity *I* had an *m*/*z* value of 159. It was confirmed in most of the degradation media but the relative concentration in respect with SSD did not have statistical relevance to show any correlation as a degradation product. The value for *m*/*z* of 158 corresponds to benzenesulfonic acid and the presence of the product ions with *m*/*z* (143, 115, 78,2 and 60.7) confirms the hypothesis.

As a function of their method of occurrence, in different degradation media, Fig. 7 shows a possible degradation pathway.

## 5. Conclusions

A good and reliable method was proposed for the identification of the impurities for silver sulfadiazine. Up to 9 possible impurities were demonstrated to be degradation products and good correlations being obtained in respect with silver sulfadiazine evolution. Additional degradation media were considered, beyond those in ICH guidelines, being in this way demonstrating other possible mechanisms of degradation under the presence of metal ions. A kinetic model for SSD degradation related to temperature and relative humidity has been proposed which could be extended to the prediction of shelf life period of the substance. Applying the method to other confirmation techniques such NMR and exact mass high resolution spectrometry could create the possibilities to introduce the method in current practical laboratory workflows.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.07.029.

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