Current Chemistry Letters 6 (2017) 167–176

Contents lists available at GrowingScience

Current Chemistry Letters

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Metronidazole citrate ester as the new prodrug of metronidazole

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C H R O N I C L E

Article history:
Received March 2, 2017
Received in revised form
June 1, 2017
Accepted June 21, 2017
Available online
June 22, 2017

Keywords:
Ames assay
Ester of metronidazole
Impurity profile
Metronidazole citrate disodium
Metronidazole prodrug

A B S T R A C T

Many attempts have been made since 1960th to obtain ester prodrugs of metronidazole active moiety to be used for parenteral forms, with the same action against microorganisms. Until now there is not any ester prodrug marketed for this route of administration. The synthesis from metronidazole and citric acid of new ester prodrug of metronidazole with citric acid in a form of disodium salt and the way of purification were described. The structure of sodium metronidazole citrate was elucidated with IR, MS, ^{1}H NMR and ^{13}C NMR spectra. Also impurities present in this ester were identified using ^{1}H NMR technique. Additionally, the solubility in water was measured as well as pH of 10% (w/w) aqueous solution, and both values indicated that there is a possibility to obtain concentrated solutions for injection of neutral pH, even without need of buffering. Finally, the Ames assay using six tester strains *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* uvrA, [pKM101] shown weak genotoxic potential, comparable with metronidazole.

1. Introduction

Metronidazole is a synthetic agent 1-(2-Hydroxyethyl)-2-methyl-5-nitroimidazole (see **Fig. 1**) used against different protozoans and bacteria.

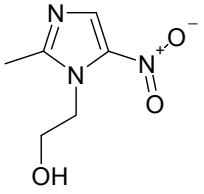

Fig. 1. Metronidazole

The mechanism of the cytotoxic action of metronidazole on anaerobic microorganisms is not well understood, but it is anticipated that derivatives arising from the reduction of the nitro group, especially the nitro anion radical R-$\dot{N}O_2^-$, are the most likely candidates for DNA damage in bacteria[1]. This hypothesis is probably a result of many experiments, which have provided evidences that DNA is

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doi: 10.5267/j.ccl.2017.6.002

susceptible to damage caused by free radicals[2]. Taking into account this possible action the prodrugs should also have a potency to form a nitro anion radical.

Metronidazole is classified by International Agency for Research on Cancer (IARC) into 2B class, i.e. possible human carcinogen, as it has shown mutagenic activity *in vitro* including positive Ames test, although studies *in vivo* have failed to demonstrate a potential for genotoxicity[3,4].

This active substance has been used for the treatment of bacterial infections for above 45 years, but still metronidazole is successfully used for the treatment of vaginosis, trichomoniasis, amoebiasis, and giardiasis, and still it is the criterion standard for therapy of anaerobic bacterial infections[5], although during last decades many new derivatives of metronidazole were investigated with antibacterial, antifungal and also *H. pylori* urease inhibitory activity.

Metronidazole is marketed as the antibacterial agent, mainly for oral route of administration, although as the active moiety it is used also in parenteral dosage forms. Since the solubility of metronidazole in an aqueous solution is only about 10 mg/mL, the way of administering a parenteral dose of metronidazole is difficult, as large volume of a drug is needed to administer a single dose of this active substance. Metronidazole for injection available for treatment is a buffered solution at the concentration of 5 mg/mL and the recommendation for treatment is 500 mg of metronidazole in 100 mL to be infused over 20 to 60 minutes.

Many attempts have been made in order to improve the solubility of metronidazole in aqueous solutions. One approach is solubilizing agents adding and here mono- or dihydroxy benzoic acid, or a mono- or dihydroxybenzyl alcohol, preferably gentisic acid were added[6]. The second approach is co-solvents adding such as N,N-dimethylacetamide/ethanol or nicotinamide/propylene glycol/2,2-dimethyl-1,3-dioxolane-4-methanol[7].

Another approach is to administer metronidazole as a pharmaceutically accepted derivative with sufficient solubility in water. And here, the simple salt – metronidazole hydrochloride has been already marketed as Flagyl®500 mg for injection, sterile for intravenous infusion. The problem with this derivative is that due to the low pH (0.5 – 2.0) it is forbidden for direct injections. This drug is to be administered by slow intravenous drip infusion only and to be reconstituted before use for the average concentration of 100 mg/mL.

Metronidazole as an alcohol undergoes esters formation. Although various ester structures have been investigated, until now metronidazole benzoate is the only one ester prodrug present on the market. *In vitro* antimicrobial activity of metronidazole benzoate against *Clostridium perfrigens* is 20 folds better than for metronidazole[8], but unfortunately its solubility in water systems is not satisfied for injections, because it is practically insoluble in water.

Historically, the first new esters of metronidazole with mono- or dicarboxylic aliphatic or aromatic acids and the synthesis of few of them (dichloroacetyl, pivaloyl, cinnamoyl, succinoyl, benzoyl, chlorobenzoyl, methoxybenzoyl, nitrobenzoyl, salicyloyl, phthaloyl) were described in 1960th. Optionally acid addition salts of the above mentioned esters, containing anions which are relatively innocuous to the organism and hydrogen were patented[9].

Among others, the esters of metronidazole as aminocarbonyloxy- or methylaminocarbonyloxy- or dimethylaminocarbonyloxy- or hydroxyaminocarbonyloxy- derivatives[10], and various sulphonic acids are also known[11]. Also the hydrochloride of an ester of metronidazole with N,N-dimethylglycine has been characterized as regards the solubility in water which was even better than metronidazole itself. Moreover it was proved that this new compound is suitable for parenteral route of administration[12]. The

design and synthesis of a series of metronidazole multi-esters having two or more metronidazole groups linked together by aryl or alkyl systems is also described, as well as metronidazole trimesate with antibacterial potential[13]. Furthermore, few amino acid esters of metronidazole as methylpiperazinoacetate, N,N-diethylglycinate hydrochloride or 4-ethylpiperazinoacetate were investigated to be considered as good candidates for water-soluble prodrug forms[14]. The esters with naturally occurring amino acids were also patented as good solution for injections[15]. Metronidazole retinoate is also known, potentially useful for acne treatment[16].

Amongst inorganic esters, the monoester of metronidazole with phosphoric acid was described[17]. Metronidazole phosphate has the solubility in water of approximately 50 folds better than metronidazole and this derivative, especially in a form of a salt, was presented as a useful prodrug of metronidazole[17,18]. Many substituted aryl esters of metronidazole were synthesized and characterized as regards its possible antiglycation activity[19]. Moreover, fourteen metronidazole esters with salicylic acid derivatives were reported and investigated *in vitro* against *Helicobacter pylori* urease[20].

To conclude, many attempts have been made, but until now there is no satisfied solution for metronidazole for injection. Although Flagyl®500 mg (metronidazole hydrochloride) is the recommended for this route of administration, it is not allowable for direct use what is caused by non-appropriate pH. The second one marketed is Metronidazole 5 mg/mL for injection according to USP monograph, and it is an aqueous solution containing metronidazole and such excipients as citric acid, dibasic sodium phosphate and sodium chloride. The problem with the last is that the recommended maximal single dose is for example 2000 mg of metronidazole for *Urogenital trichomoniasis* or *Giardiasis*, what equals long daily time of infusion from 80 minutes to even 4 hours, depending on the patient.

The goal of this study was to synthetize the potential new prodrug of metronidazole with sufficient solubility in water system, and neutral pH, and also containing the majority of components of Metronidazole 5 mg/mL for injection according to USP. This new structure is disodium salt of citric acid ester of metronidazole (see **Fig. 2**) with a molecular formula $C_{12}H_{13}N_3O_9Na_2$.

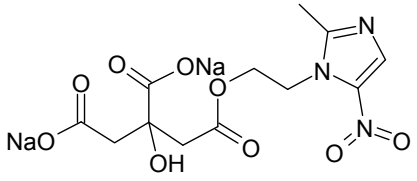


Fig. 2. Disodium metronidazole citrate

The aim of this work was also determination of the impurities profile of the new derivative, including possible by-products.

The other important fact is that metronidazole has shown mutagenic activity in a number of *in vitro* assay systems, including Ames test[21], but studies in mammals (*in vivo*) failed to demonstrate a potential for genetic damage[22]. Moreover, the only one widely marketed prodrug – Metronidazole benzoate is even more mutagenic than metronidazole in Ames mutagenicity assay using *Salmonella typhimurium* (TA-100), dose level of 0.5 μmole, 1 μmole and 5 μmoles[23]. Taking into account the *in vitro* genotoxicity of metronidazole and metronidazole benzoate, initial Ames study of the new derivative was performed to compare with the originators.

2. Results and Discussion

Synthesis of disodium metronidazole citrate

Metronidazole citrate disodium was synthesized from metronidazole and citric acid in acetonitrile as an inert solvent, and in presence of solid acid catalyst (cationite with sulphate groups, dry). Esterification catalysed by the common acid catalysts were impossible because inorganic esters were formed during the process, whereas the solid catalyst was simply removed. The crude product was initially purified from unreacted citric acid after basification in ethanol, due to the extremely various solubility of sodium citrate and disodium metronidazole citrate in this solvent. Next the substance was purified in water (dissolved, filtered and evaporated to dryness) in order to remove majority of unreacted metronidazole. The assay of metronidazole citrate disodium was determined with ^{1}H NMR (see **Table 1**).

Table 1. Assay of metronidazole citrate and content of impurities

Component	Integration	Molar ratio [mol/mol]	Weigh ratio [w/w]	Percentage content
Disodium metronidazole citrate (see **Fig. 2**)	1.000 [2 H]	1.000	345.26	93.37 95.1 (on dried basis)
Disodium metronidazole citrate isomer (see **Fig. 6**)	0.054 [2 H]	0.027	9.32	2.52
Metronidazole	0.054 [2 H]	0.027	4.62	1.25
Disodium monoethyl citrate	0.038 [2 H]	0.019	4.18	1.13
Ethanol	0.278 [2 H]	0.139	6.40	1.73

The structure was confirmed by IR (see **Fig. 3**), ^{1}H NMR (see **Fig. 4**) and ^{13}C NMR (see **Fig. 5**) techniques.

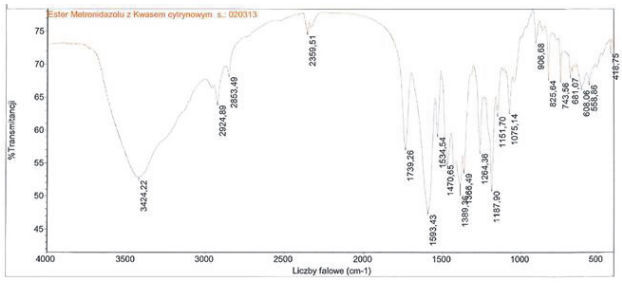


Fig. 3: IR spectrum of metronidazole citrate (tablet in KBr)

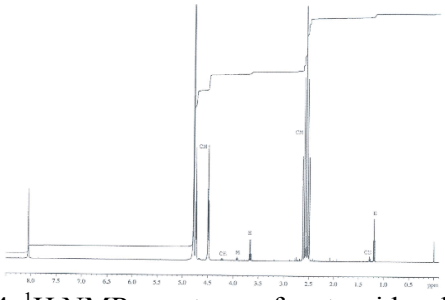


Fig. 4. ^{1}H NMR spectrum of metronidazole citrate (40 mg in 1 mL of D_2O)

M – metronidazole; CM – disodium metronidazole citrate isomer, CE – disodium monoethyl citrate, E – ethanol

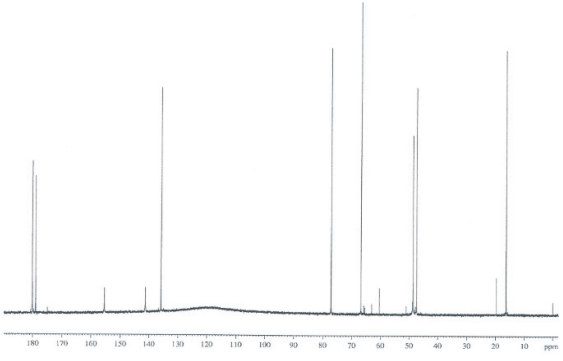


Fig. 5. ^{13}C NMR spectrum of metronidazole citrate (40 mg in 1 mL of D_2O)

Identification of impurities

The impurities of disodium metronidazole citrate were identified using ^{1}H-NMR method. The chemical shifts elucidated that amongst impurities are present: unreacted metronidazole (quartet; 4.196, 4.210, 4.224, 4.238), disodium metronidazole citrate isomer presented on **Fig. 6** (doublet; 2.746, 2.777)), disodium monoethyl citrate formed in reaction of unreacted citric acid and ethanol (triplet; 1.263, 1.278, 1.292 and triplet 3.911, 3.922, 3.932) and residual ethanol (triplet; 1.174, 1.188, 1.202 and quartet 3.638, 3.652, 3.673, 3.680). The content of impurities is presented in **Table 1**.

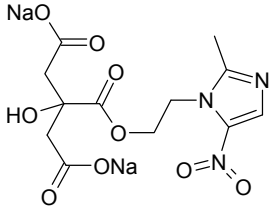


Fig. 6. Disodium metronidazole citrate isomer

There was no sufficient proof on ^{1}H NMR spectrum for presence of unreacted citric acid.

Determination of solubility in water and pH

Disodium metronidazole citrate in amount of 0.1 g was dissolved in 0.1 mL of water and clear solution was obtained. pH of 10% solution in water (without CO_2) was determined to be 5.57.

Ames assay of disodium metronidazole citrate

The determination was performed according to Instructions of Ames MPF™ Penta I Test Kits–semisolid (Xenometrix), using six tester strains – four *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and two *Escherichia coli* strains uvrA, [pKM101] exposed in mixture. Each *S. typhimurium* strain and *E. coli* mixture strain was exposed to the tested compound dissolved in DMSO. The exposure was performed in the presence and in the absence of metabolic activation system using S9 fraction. Six log-concentrations of sodium metronidazole citrate, as well as negative control (DMSO) and positive control (2-aminoanthracene) were diluted in 1:25 ratio and incubated for 90 minutes at 37°C (*E. coli* strains with S9 fraction were incubated only for 20 minutes) in triplicate. The exposure cultures were diluted, mixed and aliquot in 50 µL fractions into 48 wells of a 384-well plate and incubated for 2 days. The plates were analysed by counting the positive wells (UV 600 nm). The 'Standard Deviation of Positive Wells per Concentration' was calculated the standard deviation values for the Mean Number of Positive Wells. Student's t-test (1-sided, unpaired) was used to determine significance at the a = 0.05 level. The results of disodium metronidazole citrate to be considered as clear negative were observed on the tester strains TA98 in the presence of metabolic activation (see **Fig. 7**) ,TA1537 in the absence of metabolic activation (see **Fig. 8**), TA1537 in the presence of metabolic activation (see **Fig. 9**).

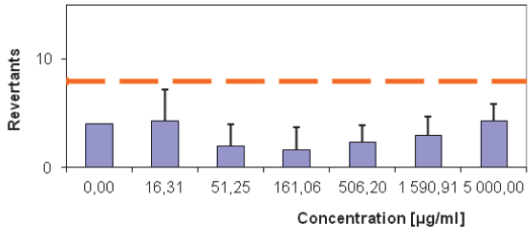


Fig. 7. Ames assay for TA98 +S9

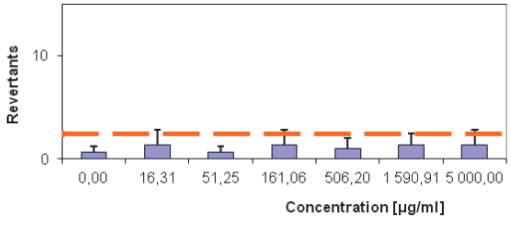


Fig. 8. Ames assay for TA1537 -S9

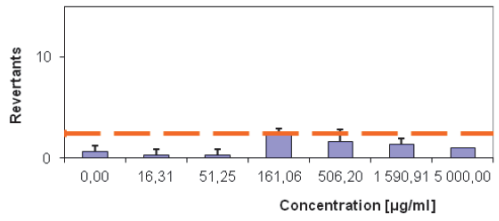


Fig. 9. Ames assay for TA1537 +S9

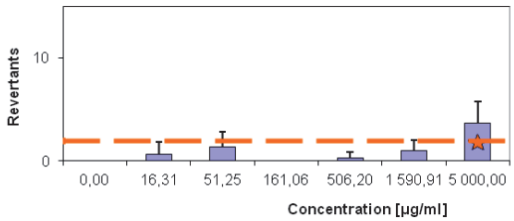


Fig. 10. Ames assay for TA98 -S9

Test results on TA98 strain in the absence of metabolic activation (see **Fig. 10**) revealed single statistically significant increase in the mean number of positive wells in the highest tested dose. Also test results on TA100 strain (see **Fig. 11**, **Fig. 12**) in the absence and in the presence of metabolic activation revealed regular statistically significant increase in the mean number of positive wells over the entire range tested.

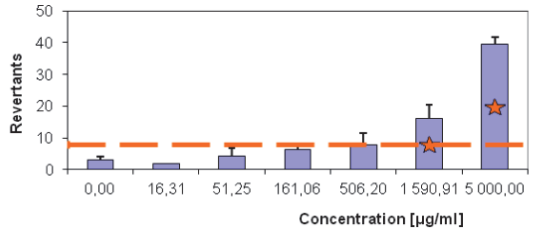


Fig. 11. Ames assay for TA100 -S9

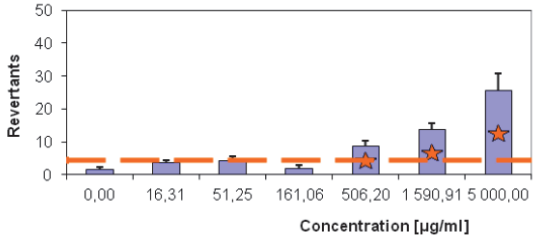


Fig. 12. Ames assay for TA100 +S9

Test results on TA1535 strain (see **Fig. 13**, **Fig. 14**) in the absence and in the presence of metabolic activation revealed single statistically significant increase in the mean number of positive wells in the highest tested doses.

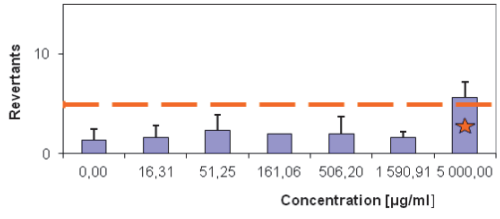


Fig. 13. Ames assay for TA1535 -S9

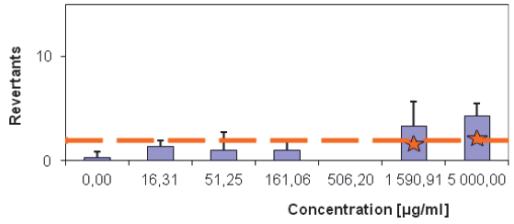


Fig. 14. Ames assay for TA1535 +S9

Test results on EC Combo mixture (wp2 [pKM101] and wp2 uvrA) in the absence and in the presence of metabolic activation (see **Fig. 15**, **Fig. 16**) revealed statistically significant (close to 2.0-fold but not exceeded it) increase in the mean number of positive wells in the highest tested dose.

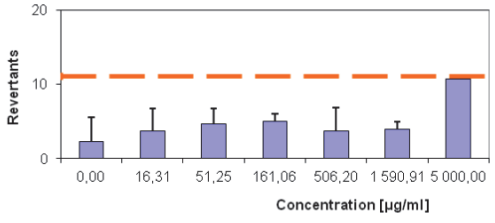


Fig. 15. Ames assay for EC Combo -S9

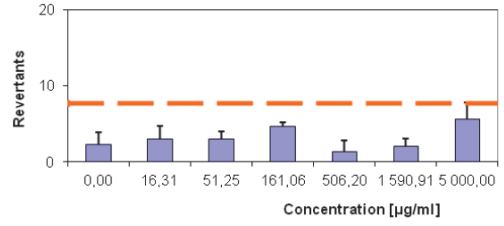


Fig. 16. Ames assay for EC Combo +S9

As the result of Ames assay, disodium metronidazole citrate was considered as weak mutagenic *in vitro*, under the condition of the assay, what may be compared to already marketed metronidazole and metronidazole benzoate. Disodium metronidazole citrate was found to induce point mutations by both frame shift and base substitution.

3. Conclusions

- The synthesis of an ester of metronidazole with citric acid is the first attempt since 1960th to obtain the mono-ester with tri-carboxylic acid. The synthesis procedure was carried out in mild conditions, using non-toxic organic solvent and solid catalyst easy removable from the reaction mixture. The unreacted substrates can be easily removed from the desired product due to strong differences in solubility in water systems. The assay of the new derivative was performed by ^{1}H-NMR technique to be 95.1% (on dried basis).
- The solubility in water (very soluble) and pH of 10% solution 5.57 of the new derivative were determined, and both values were satisfied for the drug forms for injection.
- The declared structure of disodium metronidazole citrate was elucidated by NMR (^{1}H and ^{13}C) and IR spectra.
- To facilitate impurities identification the analysis of ^{1}H-NMR spectrum was performed. The impurities were identified by chemical shifts comparison. Amongst impurities it was found unreacted metronidazole, residual solvent (ethanol) and by-product, which was disodium monoethyl citrate. Also less favourable isomer monoester of citric acid with metronidazole was formed as by-product. No traces of unreacted citric acid were identified.

Additionally, Ames assay for disodium metronidazole citrate was performed and *in vitro* results indicates weak genotoxic potential, compared to already marketed active substances as metronidazole and metronidazole benzoate. Based on the results of testing for esters of metronidazole with acetic acid, propionic acid, butyric acid, valeric acid, caproic acid, benzoic acid or phenyl cinnamic acid, where it was suggested that the antimicrobial activity increases with increase in the carbon chain length of the acid[23] it may be anticipated that the therapeutic properties of the new ester with citric acid are at least compared with metronidazole itself. It may be concluded that sodium metronidazole citrate may be a useful prodrug for parenteral drug forms containing metronidazole active moiety.

4. Experimental

4.1. Reagents and chemicals

4.1.1. Synthesis of disodium metronidazole citrate

Metronidazole was obtained from Polpharma S.A. Citric acid anhydrous, Amberlyst® 15Dry, acetonitrile, sodium hydrogen carbonate, ethanol anhydrous were purchased from Sigma-Aldrich. pH indicator was purchased from MERCK.

4.1.2. Nuclear magnetic resonance (NMR) spectroscopy analysis

D_2O 99.8% D was purchased from Armar chemicals.

4.1.3. Ames assay

Ames MPF™ Penta I kit, ver. 4.5_S January 2012 was purchased from Xenometrix, D-glucose-6-phosphate sodium salt, potassium chloride, magnesium chloride $MgCl_2 \times 6H_2O$, sodium dihydrogen phosphate NaH_2PO_4 and β-Nicotinamide adenine dinucleotide phosphate hydrate were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was obtained from BioShop, sodium chloride from Chempur and sodium hydroxide from POCH.

4.1.4. IR analysis

Potassium bromide (for IR spectroscopy Uvasol®) was purchased from Merck.

4.2. Instrumentation and conditions

4.2.1. Synthesis

Magnetic mixer Heidolph, Vacuum pump from KNF, were used for the synthesis.

4.2.2. Nuclear magnetic resonance (^1H NMR and ^{13}C NMR) spectroscopy analysis

^1H NMR and ^{13}C NMR spectra were recorded in D_2O on a Bruker Avance NMR spectrometer operating at 500,130 MHz at room temperature (20°C).

4.2.3. Ames assay

37°C dry incubator Binder, Deep frezer ThermoScientific, Environmental Shaker Edmund Bühler GmbH, Multi-mode microplate reader BioTekSynergy™, pH-meter Crison, Spectrophotometer UV Shimadzu were used for assay.

4.2.4. IR analysis

IR spectra were recorded with a Thermo Nicolet FT-IR (model iS10) spectrometer.

4.2.5. pH determination

pH were measured on Mettler-Toledo (model SevenEasy) pH-meter.

4.2.6. MS analysis

MS spectrum was recorded on High-Performance Liquid Chromatograph Prominence LC-20 (Shimadzu) coupled with tandem mass spectrometer 4000 Q TRAP (Applied Biosystems Inc, USA), equipped with an electrospray (ESI) ion source (TurboIonSpray) and the triple quadrupole/linear ion trap mass analyzer.

4.3. Synthesis procedure

5.13 g of metronidazole, 17.3 g of citric acid anhydrous and 2.57 g of Amberlyst® 15 Dry were weighted into a vessel, and 240 mL of acetonitrile was added. The suspension was boiled for 11 hours. The mixture was filtered and the filtrate was evaporated to about 80 mL under vacuum. The obtained residue was chilled and the precipitate was filtered. The filtrate was concentrated under vacuum to obtain 6.61 g of yellow oil. Then the product was dissolved in 100 mL of acetonitrile and heated to 40°C. The saturated solution of sodium hydrogen carbonate was dropped until pH 7 was reached. In the next step 250 mL of acetonitrile was dropped until suspension is formed. The precipitate was filtered and 30 mL of acetonitrile was added. The filtrate was evaporated under vacuum and 6.44 g of the powder was obtained. Anhydrous ethanol in amount of 650 mL and 2.10 g of sodium hydrogen carbonate were added. The obtained suspension was mixed and filtered. The filtrate was evaporated under vacuum to obtain 1.32 g of yellowish powder. Then the product was purified by dissolving in water, filtration and evaporation of a clear filtrate to dryness.

IR analysis (cm^{-1}): 419, 559, 609, 682, 744, 826, 907, 1075, 1152, 1188, 1264, 1366, 1389, 1471, 1534, 1593, 1739, 2359, 2853, 2925, 3424.

The results of NMR analysis are presented in **Table 2** and **Table 3**.

Table 2. ^{1}H NMR results for metronidazole citrate disodium

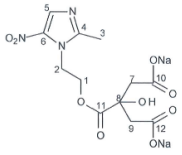
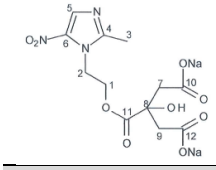
Proton	**Chemical shift, δ ppm**	
		
H-7, H-9	2.49, 2.52 2.59, 2.62	doublet of doublets
H-3	2.56	singlet
H-1	4.48, 4.49, 4.50	triplet
H-2	4.72, 4.73, 4.74	triplet
H-5	8.05	singlet

Table 3. ^{13}C NMR results for metronidazole citrate disodium

Carbon	**Chemical shift, δ ppm**
	
C-3	16.3
C-2	47.4
C-7, C-9	48.6
C-1	66.8
C-8	77.2
C-5	135.8
C-6	141.3
C-4	155.5
C-11	179.0
C-10, C-12	180.1

ESI-MS analysis has confirmed the molecular mass 345.1 g/mol of metronidazole citrate (see **Fig. 17**).

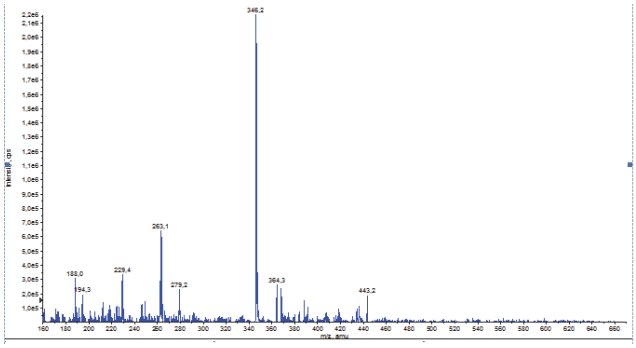


Fig. 17. ESI-MS spectrum of metronidazole citrate in the positive ion mode

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