

Detection of Impurities in Anti-infective Generic Drugs in Brazil by Liquid Chromatography-Mass Spectrometry

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Impurities found in generic medicines may contribute to loss of efficacy and adverse side effects when administered to patients suffering from various diseases. Methods of analysis of the quality of drug products are well advanced. Herein, we used Liquid Chromatography with Diode Array Detector coupled to Mass Spectrometry to detect the presence of organic impurities and determine the quantity of the Active Pharmaceutical Ingredient (API) present in representative Antibiotics (2) and Antifungals (2). Possible impurities were detected in some of the generic drugs in both classes of anti-infective drugs. No impurities were detected in the amoxicillin. The compounds 3'-N,N-Di(dimethyl) azithromycin (azithromycin impurity E) and 3'-De(dimethylamino)-3'-keto azithromycin (azithromycin impurity N) were detected in generic azithromycin. For itraconazole, the compounds cis-[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-4-yl-methyl)-1,3-dioxolan-4-yl]methylmethanesulfonate and trans-[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl-methyl)-1,3-dioxolan-4-yl]methylmethanesulfonate, besides a third substance identified as 2-(2-Butyl) -4-{4-[4-(4-methoxy-phenyl)-piperazin-1-yl]-phenyl}-2,4-dihydro-[1,2,4]-triazol-3-one were detected as possible impurities. Interestingly, an additional peak was noted in the chromatogram for the generic fluconazole, in addition to the peak of the API; however, none of known impurities of fluconazole were identified. We conclude that tests in addition to bioequivalence measurements may be required to assess post-market generic quality. Such surveillance of generic quality should be performed routinely.

Keywords: Generic Drugs. Mass Spectrometry. Drug Impurities.

Impurities may be present in medicinal products generated from poorly controlled manufacturing conditions, poor-quality substrates and/or API synthesized by a process different from than the certified synthesis pathway [1,2]. The impact of the use of low-quality drugs is related to the adverse effects caused by the presence of undesirable substances, such as impurities and worse, it may contain the incorrect Active Pharmaceutical Ingredient (API) [3].

The adulteration of drugs has been grouped into five categories: (i) copies of the authentic medicine, but with changes in the quantity of API; (ii) products with incorrect API, which may be of

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inferior quality; (iii) preparations with an absence of API; (iv) medicines with very high or very low API content; (v) contamination with known and/or unknown impurities [4,5].

Due to lower costs, the use of generic drugs has been promoted in many countries [6]. Policies to reduce costs and increase the availability of medicines have contributed to the prevention of drug shortages, in addition to improving accessibility to drugs in various therapeutic classes, especially in developing countries [7-9]. Analytical methods for rapid and accurate drug testing are necessary to combat the increasing number of adulterated drugs as well as sophisticated counterfeits [10-15]. The deployment of such analytical technology will have an important role in detecting drug tampering. The safety of medicines depends on the absence of impurities and accurate API levels. Effective and reproducible methods for detecting drug impurities and API levels may provide insights into the quality of generic drugs available in the global marketplace [16,17].

High-performance liquid chromatography (HPLC) has been a common technique for the determination of impurities in pharmaceutical products [11]. For basic identification and quantification of the components of the sample, UV/Vis spectrophotometry data is gathered using a diode array detector (DAD). Additionally, this analytical technique can be coupled to mass spectrometry (MS) for the structural identification of components of interest such as impurities resulting from improper synthesis [18,19]. Thus, chromatographic techniques have been powerful tools in the analysis and characterization of adulterated medicines because they assess the composition of the analyzed sample [10].

Herein we analyzed the generic antibiotics amoxicillin trihydrate, azithromycin dihydrate, and the generics antifungals fluconazole and itraconazole by high-performance liquid chromatography to detect the presence of impurities in the products.

Generic drugs were provided by the Minister of Health of Brazil (MH). The generic medicines provided were selected from batches stored at the MH that were ready to be distributed to the population. Each generic and branded drug tablet or capsule was prepared for HPLC-DAD-MS analysis by extracting API in methanol, with the exception of amoxicillin trihydrate (in water). The analyses were tested in collaboration with other laboratories at a referral laboratory in Boston, MA - USA (personal communication from Elucida Research). Certified standards of the API for the antibiotics (amoxicillin trihydrate and azithromycin dihydrate), were purchased from Toronto Research Chemicals (Toronto, Canada) for comparative analysis.

API Impurity and Quantification Analysis were performed as follows: Stock solutions were prepared at 1-5 mg mL⁻¹ in an aqueous or organic solvent to match the corresponding sample preparation for each compound. Analytical curves for each compound were constructed at the following concentrations: 0.05, 0.1, 0.2, 0.25 and 0.5 mg mL⁻¹.

Tablets were crushed using an Agate mortar and pestle and solubilized in an aqueous or organic solvent at 1 – 5 mg mL⁻¹, depending on the nature

of the API. For products delivered in gelatin capsule form, the two halves of the capsule were separated and the contents were solubilized in aqueous or organic solvent. Solutions were stirred at least 4 hours to ensure uniform distribution of material. The active pharmaceutical ingredient (API) was then separated from insoluble excipient by centrifugation at room temperature for 30 minutes. The supernatant (containing the API) was then removed and stored at 4°C before HPLC-DAD-MS analysis.

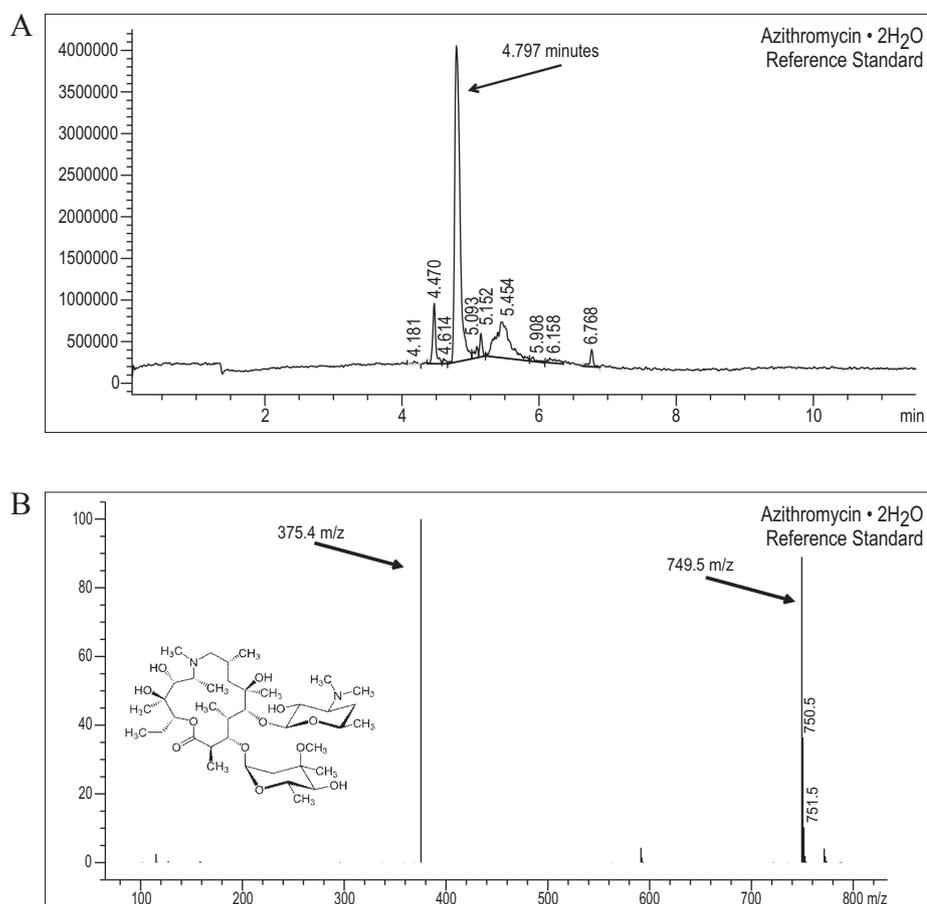
Each sample was diluted down to a nominal concentration of 0.2 or 0.5 mg mL⁻¹, and then the actual concentration was determined based on the analytical curve. Results were reported as a percentage of expected concentration compared to standard solutions as well as compared to branded formulations when applicable. For impurity analysis, samples were run at 0.5 mg mL⁻¹.

Samples and standard solutions were run on an Agilent 1260 Series LC, equipped with a diode array detector (DAD) and an Agilent 6120B MSD for mass spectrometry analysis. All samples were run on a Poroshell 120 EC-C18 4.6 X 100 mm 2.7 µm column. For the evaluation of impurities, the drug samples were analyzed using the mass spectrometric detector, described above, in SCAN mode (m/z 100 to 800). For the quantification of the APIs, the diode array detector (DAD) was operated at the wave lengths according to the optimal absorbance of electromagnetic radiation for each analyte.

Figure 1A shows the chromatograms and mass spectrum for the pure azithromycin dihydrate standard. In Figure 1A, the peak at 4.797 min corresponds to azithromycin, as evidenced by the characteristic mass spectrum and associated molecular weights (Figure 1B). The molecular weight of azithromycin is 749 g mol⁻¹.

Figure 2A shows the LC-MS analysis, where the peak at 4.832 min corresponds to generic azithromycin while the 6.052 min peak corresponds to a potential impurity not seen in the standard formulation. In Figure 2B, we show the mass spectrum analysis of this peak, which

Figure 1. Chromatogram obtained by LC-MS (SCAN mode) for pure azithromycin dihydrate (A) and mass spectrum of pure azithromycin dihydrate (B).



revealed the characteristic of the mass-to-charge ratio associated with azithromycin, which also matches the pattern of the standard azithromycin. The molecular weight of azithromycin is 749 g mol⁻¹, as shown in Figure 2B. Also, in Figure 2A, we noted several smaller peaks, which were also seen in the standard formulation.

The peak seen at 6.052 min (Figure 2A) did not have a corresponding peak in the standard formulation. Analysis of the mass spectrum of this peak (Figure 3) revealed a chemical entity with a molecular weight around to 720 g mol⁻¹. According to Chang et al. (2015) (20), there are two possible impurities in the azithromycin with similar molecular weight. One of the molecules, azithromycin EP impurity E (3'-N,N-Di(dimethyl) azithromycin), has a molecular weight of 720.9 g

mol⁻¹ (Figure 4A). Another molecule, azithromycin EP impurity N (3'-De(dimethylamino)-3'-keto azithromycin), has a molecule weight of 719.9 g mol⁻¹ (Figure 4B).

The reference and generic formulations of amoxicillin hydrated were analyzed for impurities using LC-MS as previously described. The amoxicillin trihydrate standard (2.992 min) and both the reference (2.985 min) and generic (2.982 min) amoxicillin trihydrate show a similar peak as shown in Figure 5. The mass spectrum of the standard, the reference and the generic confirmed that the only peak observed in both chromatograms correspond to amoxicillin hydrated. Nevertheless, no other peaks were noted. Thus, it confirms that no impurities were encountered in both reference and generic amoxicillin.

Figure 2. Chromatogram obtained by LC-MS (SCAN mode) for generic azithromycin dihydrate (A) and the mass spectrum of 4.832 min peak for generic azithromycin dihydrate (B)

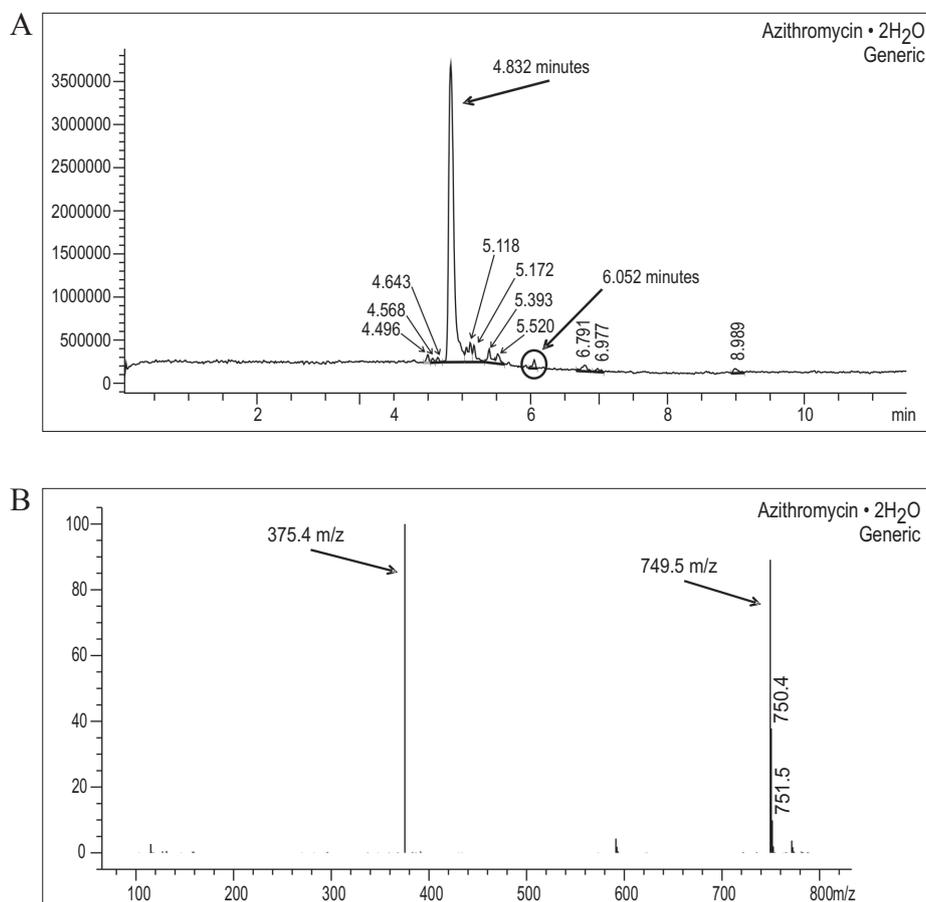


Figure 3. Mass spectrum of 6.052 min peak for generic azithromycin dihydrate. The pattern of this mass spectrum indicates a chemical entity with a molecular weight around 720 g mol⁻¹ that was not seen in the standard formulation.

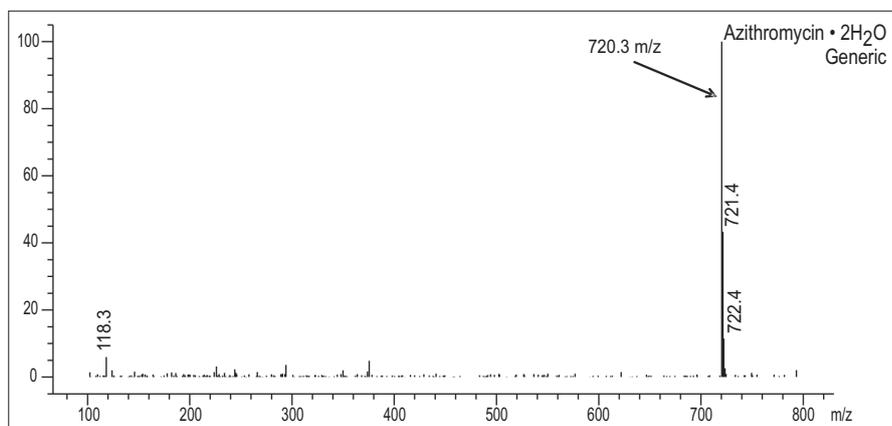


Figure 4. Chemical structure of possible impurities found in the generic formulation of azithromycin dihydrate. (A) 3'-N,N-Di(desmethyl) azithromycin (CAS#: 612069-27-9, azithromycin EP impurity E), which has molecular weight of 420.9 g mol⁻¹ and (B) 3'-Des(dimethylamino)-3'-keto azithromycin (CAS#: 612069-25-7, azithromycin EP impurity N), which has molecular weight of 419.9 g mol⁻¹.

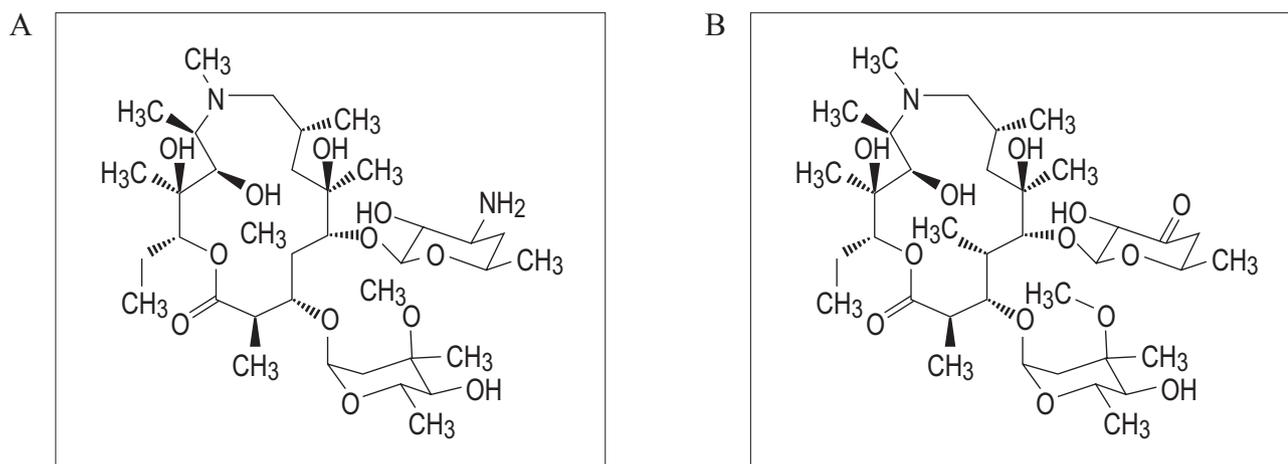
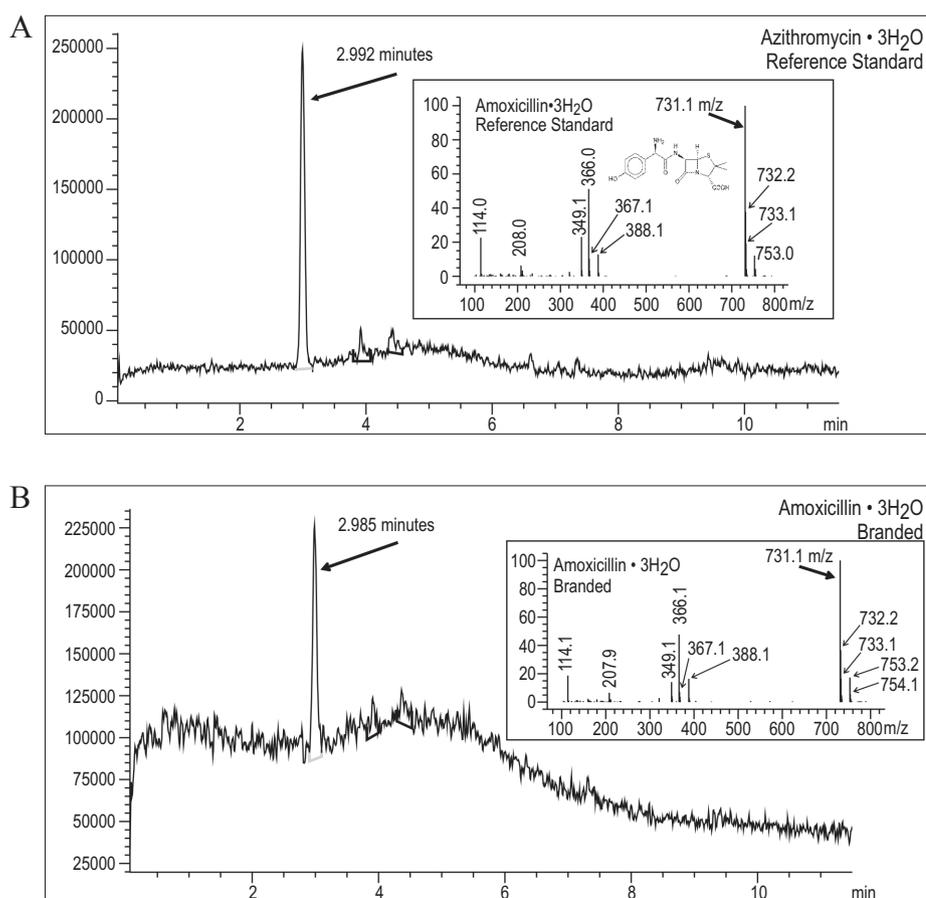


Figure 5. Chromatograms and mass spectrums obtained for (A) standard amoxicillin trihydrate, (B) reference amoxicillin trihydrate and (C) generic amoxicillin trihydrate.



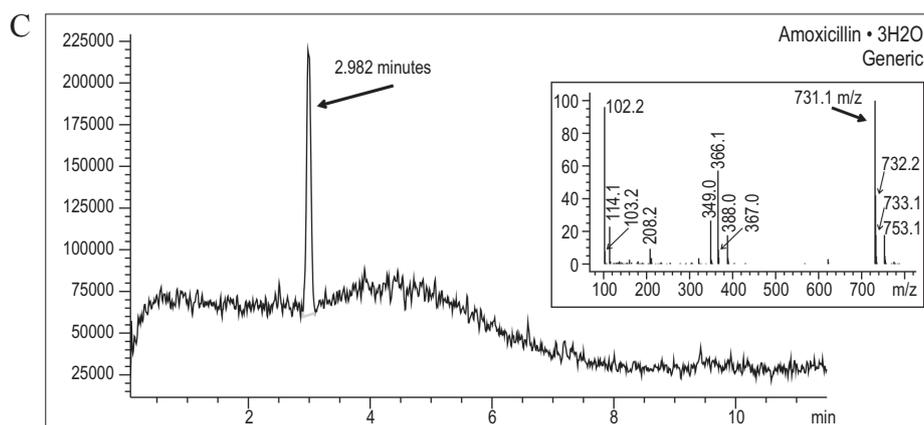
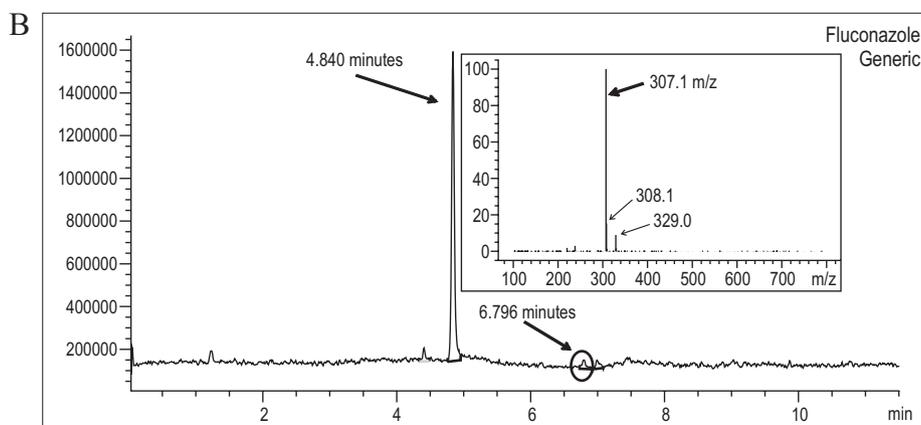
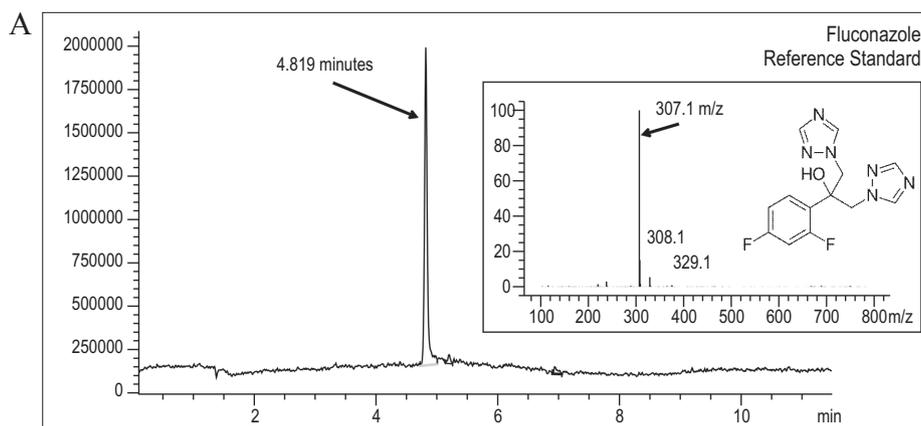


Figure 6 shows the chromatogram (LC-MS) and mass spectra associated with the standard and generic fluconazole.

Figure 6. Chromatogram obtained by LC-MS (SCAN mode) and mass spectra for standard fluconazole (A) and generic fluconazole, which the main peak corresponds to fluconazole (4.840 min) (B).



The peak at 4.840 min corresponds to fluconazole, as evidenced by the characteristic mass-to-charge ratio associated with fluconazole seen in the mass spectrum of this peak (Figure 6), in addition to the comparison with the pure standard of this substance. Additionally, there is a small peak at 6.796 min, which did not have a matching peak

in the pure standard. Further analysis of the mass spectrum of this peak (Figure 7) shows a fragment with a molecular weight of 227 g mol⁻¹. A search of known impurities of fluconazole did not reveal any molecules with the same molecular weight, but it should be noted that this does not mean that any of the known impurities are not present.

Figure 7. Mass spectrum of 6.796 min peak for generic fluconazole.

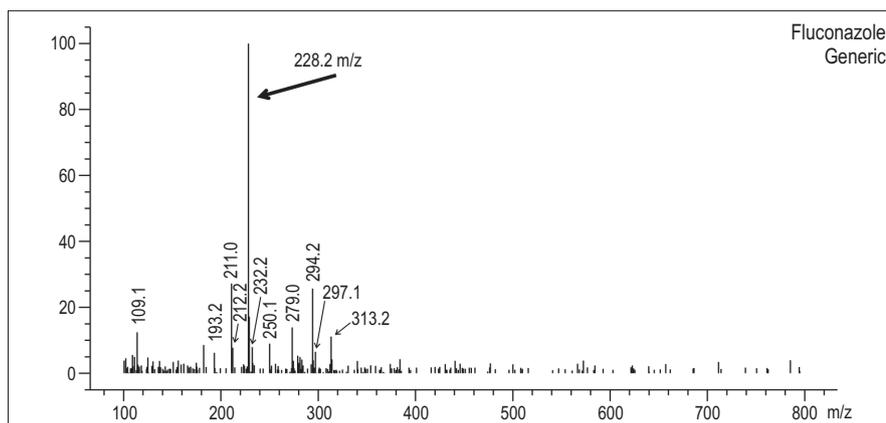
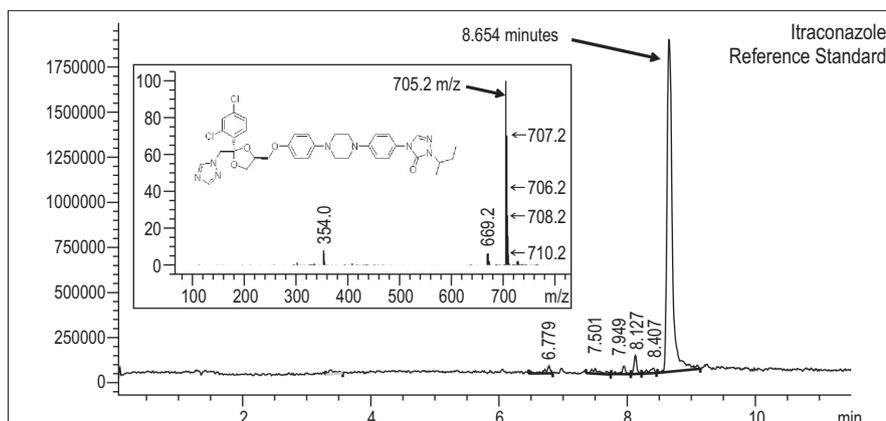


Figure 8 shows the mass spectrum for the pure itraconazole standard, which corresponds to the peak at 8.654 min from LC-MS chromatogram. The molecular weight of itraconazole is 705.65 g mol⁻¹. There are also several peaks that differ only by one mass unit: 706.2, 707.2, 708.2, and 710.2 m/z, which indicate that itraconazole can

ionize in several different ways, only differing by the mass of a few hydrogen atoms. It is essential to know when identifying possible impurities – the molecular weight indicated by a mass spectrum is not guaranteed to be the actual molecular weight of that particular chemical entity.

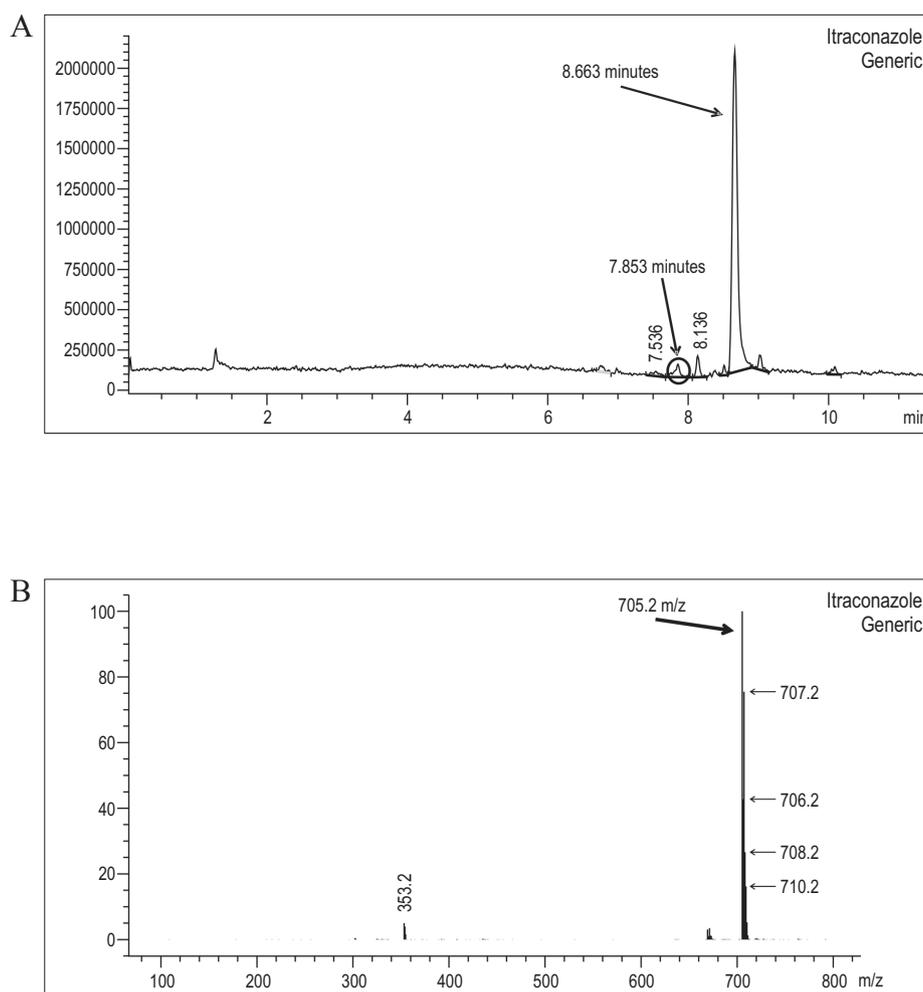
Figure 8. Chromatogram obtained by LC-MS (SCAN mode) and mass spectra for standard itraconazole.

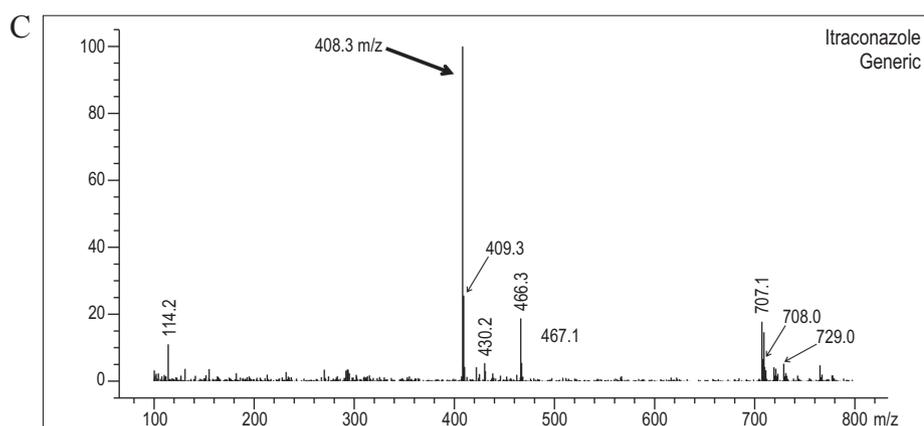


For the generic itraconazole, the peak at 8.663 min corresponds to itraconazole, as evidenced by the characteristic mass-to-charge ratios and pattern associated with itraconazole seen in the mass spectrum of this peak (Figure 9). There are several other small peaks (with retention times shown) that indicate chemical entities that have conjugated moieties. In particular, there is a small peak at 7.853 min on the LC-

MS scan, which does not have a matching peak in the standard. Analysis of the mass spectrum of this peak (Figure 9C) reveals the presence of a chemical entity with a primary fragment with a mass-to-charge ratio of 408.3 m/z. As mentioned earlier, it is unclear whether this corresponds to a molecular weight of 407 g mol⁻¹ or 408 g mol⁻¹ based on how itraconazole ionizes with ESI.

Figure 9. Chromatogram obtained by LC-MS (SCAN mode) for generic itraconazole (A), Mass spectrum of 8.663 min peak for generic fluconazole (B) and Mass spectrum of 7.853 min peak for generic fluconazole (C).

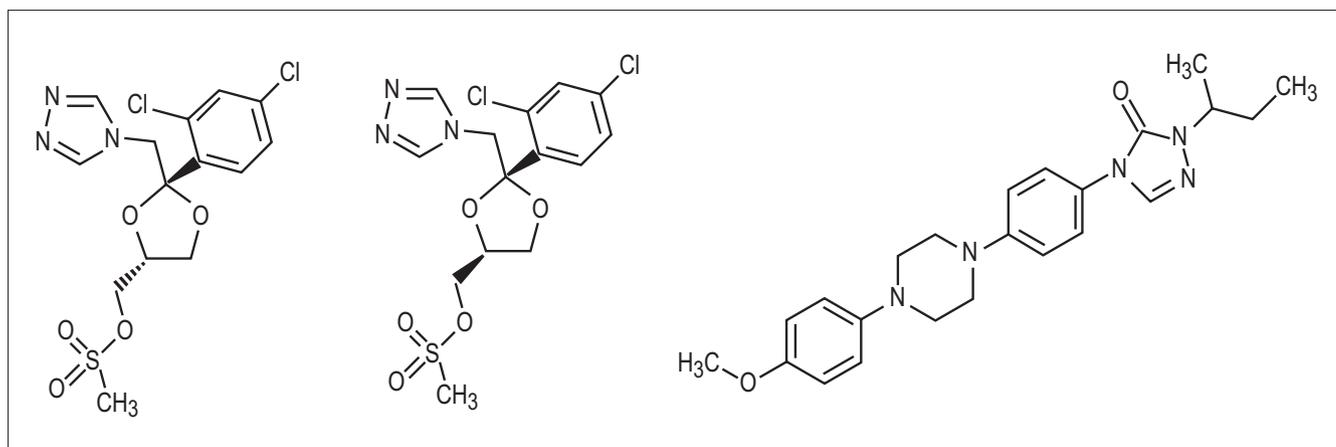




Wharton et al. in 2014 found different molecules with the mentioned molecular weights may be known impurities of itraconazole (21). Two molecules (Figure 10 A and B) are cis-trans isomers: cis-[2-(2,4-Dichlorophenyl)-2-(1H-1,2,4-triazol-4-yl-methyl)-1,3-dioxolan-4-yl]methylmethanesulfonate, and trans-[2-(2,4-Dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl-methyl)-1,3-dioxolan-4-yl]

methylmethanesulfonate, respectively. Both of these molecules have a molecular weight of 408.3 g mol⁻¹. A third molecule (Figure 10C) is a known impurity according to the European Pharmacopoeia (itraconazole EP impurity A). This molecule is 2-(2-Butyl)-4-{4-[4-(4-methoxyphenyl)-piperazin-1-yl]-phenyl}-2,4-dihydro-[1,2,4]-triazol-3-one (CAS#: 252964-68-4) and has a molecular weight of 407.5 g mol⁻¹.

Figure 10. Structure of (A) cis-[2-(2,4-Dichlorophenyl)-2-(1H-1,2,4-triazol-4-yl-methyl)-1,3-dioxolan-4-yl]methylmethanesulfonate (CAS#: 67914-86-7); (B) trans-[2-(2,4-Dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl-methyl)-1,3-dioxolan-4-yl]methyl methanesulfonate (CAS#: 854372-78-4) and (C) 2-(2-Butyl)-4-{4-[4-(4-methoxyphenyl)-piperazin-1-yl]-phenyl}-2,4-dihydro-[1,2,4]-triazol-3-one (CAS#: 252964-68-4), known impurities of itraconazole.



These data indicate that the generic itraconazole appears to contain an impurity not found in a standard formulation of itraconazole, which has a prominent mass fragment of 408.3 m/z.

Given the results discussed above, Table 1 shows a summary of the presence of organic impurities in generic and reference Brazilian medicines, as well as the evaluation of the API content present in each of the generic drugs analyzed.

Conclusion

We conclude that impurities which can interfere with the safety and efficacy of drugs are not routinely analyzed prior to registration for the treatment of human diseases. It is critical to explore the influence of such generic impurities with respect to their efficacy in addition to a bioequivalence test.

Table 1. Summary of the results of identification of impurities and quantification of the API of generic medicines compared to reference medicines.

Therapeutic Class	Compound Description	Sample Impurity*	Possible	% API vs Branded*
Antibiotic	Amoxicillin trihydrate	Reference	ND	n/a
		Generic 1	ND	97.7
	Azithromycin dihydrate	Generic 1	Azithromycin EP impurity E, N	n/a
Antifungal	Fluconazole	Generic 1	Unknown (mass ~ 227 g mol ⁻¹)	n/a
Itraconazole	Generic 1		cis-trans isomers of known impurity	n/a

*ND = not detected; n/a = not applied.

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