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Short communication

# LC–MS characterization of trace impurities contained in calcium folinate

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**Abstract**

An impurity present in calcium folinate ($N^5$-formyl-5,6,7,8-tetrahydrofolic calcium salt) active pharmaceutical ingredient (API) was detected by high-performance liquid chromatography (HPLC). Through analysis by HPLC coupled with atmospheric-pressure chemical-ionization mass spectrometry (APCI-MS), a structure for this impurity was postulated and then proved by chemical synthesis.


*Keywords:* Calcium folinate; High-performance liquid chromatography; Active pharmaceutical ingredient

## 1. Introduction

Guidelines concerning technical requirements for the registration and commercialization of pharmaceutical products in Europe, USA and Japan are published on a regular basis by the "International Conference on Harmonization" (ICH).

Since the presence of impurities in active pharmaceutical ingredients (API) can have a significant effect on their quality and safety, a guideline is devoted to this important aspect ("Impurities in New Drug Substances") [1]. In this document it is stated that impurities present in an API arising either from the synthetic procedure or during the storage (=degradation products) have to be identified and qualified. A reporting threshold of 0.1% is usually required for impurities present in API[1]. In order to comply with these regulatory requirements, it is therefore essential that the pharmaceutical industry rely on a powerful technique for the monitoring, characterization and identification of impurities present in API. It has been shown that a successful technique for solving this problem is the coupling of high-pressure liquid chromatography (HPLC) and mass spectrometry (MS) with different detection modes [2].

Most pharmaceutical compounds are polar enough to be analyzed by atmospheric pressure chemical ionization (APCI) techniques, as the molecules can either be protonated or deprotonated leading to the molecular ion. Further information on the structure of unknown impurities can be derived from fragmentation data generated by MS/MS.

In this study, the analyses were carried out on (*6RS*)-calcium folinate, also known as leucovorin calcium ("CaF"), a derivative of folic acid (Fig. 1). This molecule is a natural compound occurring in living cells, where it plays a primary role in several metabolic pathways [3]. It is the precursor of other reduced folates acting as coenzymes and carrying an "activated $C_1$-unit", which is displaced onto an appropriate substrate in the biosynthesis of nucleic acids and of a proteinogenic amino acid. CaF in the pharmaceutical industry is a drug used as: (i) vitamin against anemia, during pregnancy, in geriatrics and in case of dihydrobiopterine-reductase deficiency; (ii) oncological during the so-called "rescue therapy" after high doses of methotrexate or as modulator of the efficacy of the 5-fluoro-uracyl. LC/MS/MS analyses were

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[1] Actually, this threshold is also dependent from the "maximum daily dose" of the considered pharmaceutical product; see [1] for a detailed treatment of this topic.

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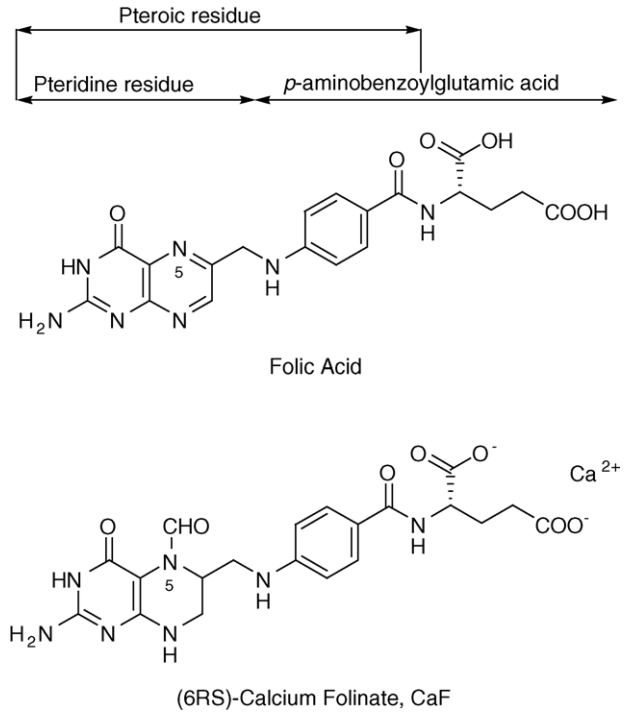



Fig. 1. Chemical structure of folic acid and (*6RS*)-calcium folinate or CaF.

performed to identify a small amount of an impurity (less than 0.05% by HPLC) generated during the CaF production. Through analytical data it was possible to formulate a hypothesis on the structure of such molecule. A synthesis of this impurity was developed to reach a definitive proof.

## 2. Experimental

Solvents and reagents were reagent-grade, purchased from commercial suppliers, and used without further purification unless otherwise stated. Evaporation in vacuo was conducted at $H_2O$ aspirator pressure. All products were dried under high vacuum (h.v., $10^{-2}$ Torr) before analytical characterization. Column chromatography (CC); $SiO_2$ 60 (40–63 μm) from *Fluka*, 0–0.3 bar pressure. TLC: $SiO_2$ *60 $F_{245}$*, *Merck*, visualization by UV light at 254/356 nm. mp: (uncorrected) were measured with a *Büchi 510 melting point* (*Büchi*, Switzerland). IR spectra [$cm^{-1}$]: *Perkin-Elmer Paragon-1000* spectrometer. NMR spectra ($^1H$, $^{13}C$): *Bruker DRX-400*, *Bruker DRX-500*; spectra were recorded at r.t. with solvent peak as reference. LC/MS experiments were performed using an *LCQ DECA* ion trap mass spectrometer equipped with an electrospray ionization (ESI) ion source, controlled by *Xcalibur* software 1.1 (*Thermo-Finnigan*, San Jose, CA, USA) and coupled to a *Spectra SYSTEM* high performance liquid chromatographic system consisting of a LC pump *P4000*, UV detector *UV2000* and Autosampler *AS3000*. HPLC experiments were carried out using a *Hypersil ODS* 5 μm 100 mm × 2.1 mm column, injection volume 10 μL, solvents are shown in the table below.

| Time (min) | Flow (ml/min) | $H_2O$, $CH_3COONH_4$ 0.005 M% | Acetonitrile (%) |
|---|---|---|---|
| 0 | 0.2 | 98 | 2 |
| 9 | 0.2 | 98 | 2 |
| 25 | 0.2 | 82 | 18 |
| 31 | 0.2 | 98 | 2 |

LC/MS experiments were carried out in positive ion mode under constant instrumental conditions: source voltage 5.0 kV, capillary voltage −14 V, sheet gas flow 35 (arbitrary units), capillary temperature 200 °C, tube lens voltage −5 V. LC/MS/MS spectra were obtained by collision induced dissociation (CID). Experiments in the ion trap were performed with an isolation width of 3 amu (*m*/*z*), the activation amplitude was around 30% of ejection RF amplitude that corresponds to 1.35 V. Elemental analyses were performed by the *Microlabor* at the *Laboratorium für Organische Chemie, ETH-Zürich*.

### *2.1. 1-{2-N-[2-(Trimethylsilyl)ethoxycarbonyl]pteroyl}-imidazole (**3**)*

A suspension of pteroic acid (2.5 g, 8.0 mmol), $Et_3N$ (4.46 ml, 32.0 mmol) and CDI (5.2 g, 32 mmol) in DMSO (40 ml) was stirred at room temperature for 4 h. To the resulting solution was added 2-(trimethylsilyl)ethanol (9.15 ml, 64 mmol), and the solution was stirred at the same temperature for 20 h. The reaction mixture was poured into a mixture of water (225 ml), AcOH (8 ml) and $Et_2O$ (160 ml). The resulting yellow precipitate was collected by filtration and dried. CC ($SiO_2$; $CH_2Cl_2 \rightarrow CH_2Cl_2$/MeOH 9:1) provided **3** (2.6 g, 64%). Yellow solid. mp >300 °C. IR (neat): 3378w, 2954s, 1689s, 1603s, 1462s, 1240s, 1173s, 1064s. $^1H$ NMR (400 MHz, DMSO-$d_6$): $\delta$ 11.75 (br. s, 2H), 8.89 (s, 1H), 8.15 (s, 1H), 7.68 (t, 1H, $J$ = 6.1 Hz), 7.62 (d, 2H, $J$ = 8.9 Hz), 7.10 (s, 1H), 6.77 (d, 2H, $J$ = 8.9 Hz), 4.65 (d, 2H, $J$ = 6.1 Hz), 4.30–4.26 (m, 2H), 1.07–1.01 (m, 2H), 0.05 (s, 9H). $^{13}C$ NMR (400 MHz, DMSO-$d_6$) 165.02, 159.26, 159.15, 154.90, 154.65, 153.05, 151.42, 149.42, 138.11, 132.68, 130.40, 129.45, 118.71, 117.61, 111.54, 64.55, 45.55, 17.12, −1.45. ESI-MS ($CH_3OH$) $ESI^+$: *m*/*z* 507 ($M + H^+$), 439 ($M - Im^+$); $ESI^-$: 505 ($M - H^-$). Anal. Calc. for $C_{23}H_{26}N_8O_4Si$ (506.59): C 54.53, H 5.17, N 22.11; found: C 54.41, H 5.25, N 22.01.

### *2.2. 1-{2-N-[2-(Trimethylsilyl)ethoxycarbonyl]pteroyl}-L-glutamine (**4**)*

A mixture of **3** (1.216 g, 1.2 mmol), L-glutamine (0.526 g, 3.6 mmol) and MTBD (1.32 ml, 7.2 mmol) in DMSO (20 ml) was stirred at room temperature for 48 h. The resulting mixture was poured into a mixture of aqueous 1 M AcOH (480 ml), MeOH (160 ml) and $CH_2Cl_2$ (480 ml), and the

whole was partitioned. The organic layer was washed with aqueous 1 M AcOH and MeOH (1:1, 320 ml). The organic layer was dried ($Mg_2SO_4$), filtered and evaporated. The residue was washed with $Et_2O$ and dried under vacuum to give **4** (0.533 g, 76%). Yellow solid. mp > 300 °C. IR (neat): 3198w, 1606m, 1515s, 1457s, 1127m, 1045s. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 11.75 (br. s, 2H), 8.83 (s, 1H), 8.21 (br. m, 1H), 7.65 (d, 2H, $J$ = 8.7 Hz), 7.31 (s, 1H), 7.01 (t, 1H, $J$ = 5.9 Hz), 6.79 (s, 1H), 6.65 (d, 2H, $J$ = 8.7 Hz), 4.59 (d, 2H, $J$ = 5.9 Hz), 4.27–4.31 (br. m, 2H), 2.17 (t, 2H, $J$ = 7.5 Hz), 2.06–2.00 (m, 2H) 1.90–1.85 (m, 2H), 1.07–1.02 (m, 2H), 0.05 (s, 9H). $^{13}$C NMR (400 MHz, DMSO-$d_6$) 182.01, 165.44, 159.30, 159.66, 154.94, 154.65, 153.05, 151.42, 149.42, 138.11, 132.68, 130.40, 129.45, 118.71, 117.61, 111.54, 64.55, 45.55, 17.12, −1.45. ESI-MS ($CH_3OH$) ESI$^+$: $m/z$ 607 ($M + Na^+$), 585 ($M + H^+$); ESI$^-$: 583 ($M - H^-$). Anal. Calc. for $C_{25}H_{32}N_8O_7Si{\cdot}H_2O$ (602.67): C 49.82, H 5.68, N 18.59; found: C 49.56, H 5.49, N 18.30.

### 2.3. *Pteroyl*-L-*glutamine* (*5*)

A mixture of **4** (0.596 g, 1 mmol) and TBAF 1 M in THF (5 ml) in DMSO (10 ml) was stirred at room temperature for 12 h. After addition of AcOH (12.5 ml), the mixture was poured into a mixture of $CH_2Cl_2$ and AcOEt (4:1, 250 ml), and the precipitate was collected by filtration and dried under vacuum to give **5** (0.361 g, 82%). Yellow solid. mp >300 °C. IR (neat): 3308w, 2964m, 1644m, 1606s, 1509m, 1079s, 1010s. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 8.45 (s, 2H), 7.65 (d, 2H, $J$ = 8.7 Hz), 6.65 (d, 2H, $J$ = 8.7 Hz), 4.59 (s, 2H), 4.27–4.35 (m, 1H), 3.18–3.25 (m, 1H), 2.25–2.35 (m, 2H), 2.10–2.20 (m, 1H), 1.97–2.18 (m, 1H). $^{13}$C NMR (400 MHz, DMSO-$d_6$) 185.17, 182.06, 172.41, 165.02, 159.26, 159.15, 156.94, 154.65, 153.05, 151.62, 149.42, 138.11, 131.72, 130.40, 123.14, 118.71, 117.61, 111.54, 64.55, 36.95, 31.20, 19.48. ESI-MS ($CH_3OH$) ESI$^+$: $m/z$ 441 ($M + H^+$); ESI$^-$: 439 ($M - H^-$). Anal. Calc. for $C_{19}H_{20}N_8O_5{\cdot}2H_2O$ (476.44): C 47.89, H 5.07, N 23.51; found: C 47.58, H 5.18, N 23.31.

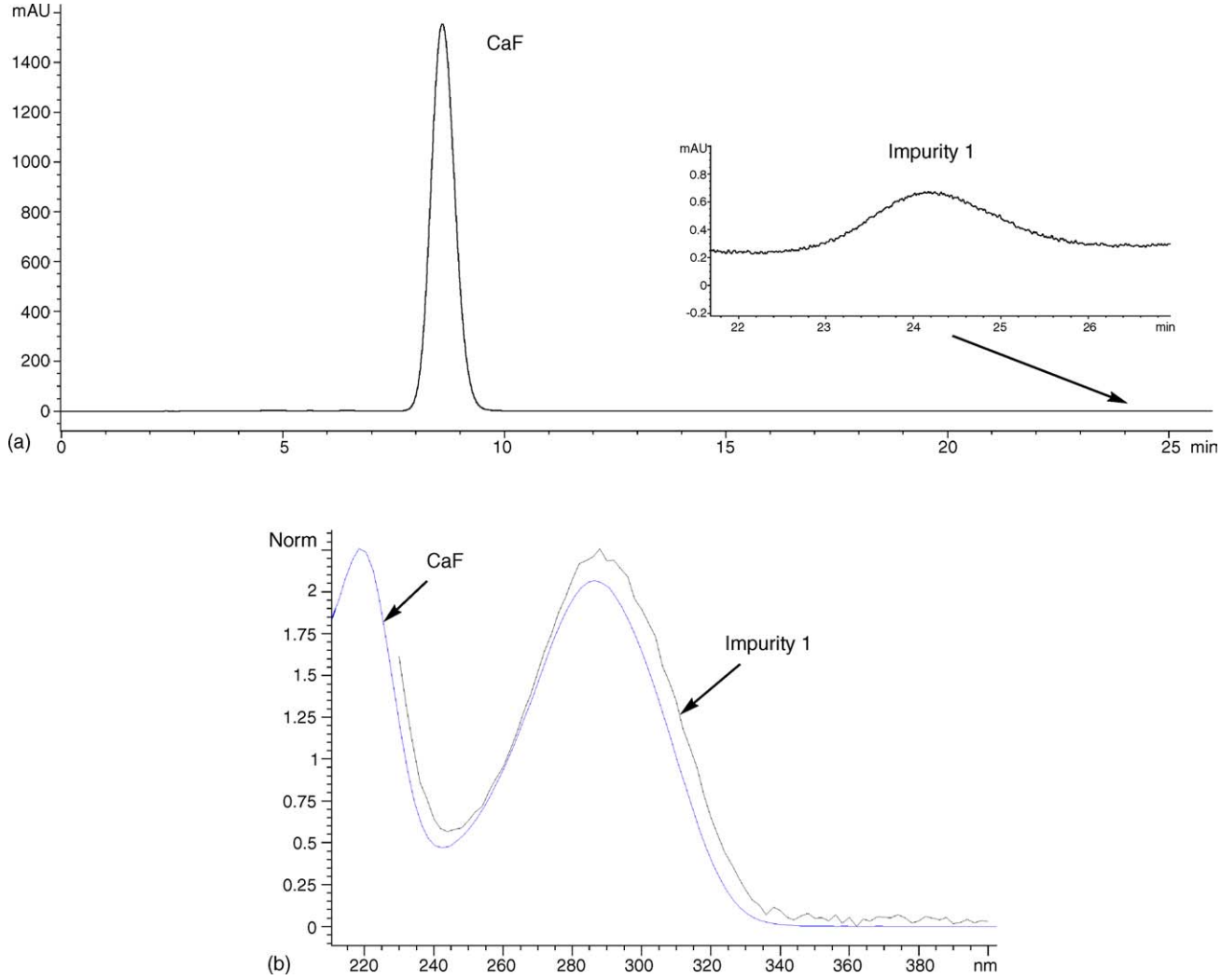



Fig. 2. (a) HPLC-UV (DAD) chromatogram of calcium folinate with UV detection at 280 nm in absorbance units (mAU); inset magnification of impurity **1** HPLC chromatogram. (b) Comparison between UV spectra of CaF and impurity **1**.

### 2.4. $N^5$-formyl-tetrahydropteroyl-L-glutamine (impurity **1**)

Under Ar, to 80 ml of degassed water **5** (0.500 g, 1.13 mmol) was added, then 20% NaOH to reach a pH value of 8.3 (complete dissolution). $NaBH_4$ (0.128 g, 3.39 mmol) dissolved in 10 ml of degassed water was added to the reaction vessel and the mixture stirred for 3 h. The pH of the mixture was increased to 3.5 with 18% HCl and the resultant suspension was centrifuged (10 min; 5000 rpm). The resultant solid was collected with 30 ml of degassed acetone and dried under reduced pressure. To the dried product, 4 ml of anhydrous and degassed DMSO and 25 ml of anhydrous and degassed formamide were added. The suspension was heated at 70 °C under reduced pressure (20 mbar) for 48 h. EtOH/(200 ml) was added to the solution and the solid obtained was collected by filtration and then dried under reduced pressure. Yellow/white solid. mp 281–285 °C. IR (neat): 3324w, 2946m, 1714s, 1602s, 1493m, 1369s, 1247s, 1216s, 1192s, 1041s. $^1$H NMR (500 MHz, $D_2O$): $\delta$ 8.77 (s, 1H), 7.62 (dd, 2H, $J$ = 8.05 Hz), 6.70 (dd, 2H, $J$ = 5.9 Hz), 4.82 (m, 1H), 4.32 (m, 1H), 3.56 (m, 1H), 3.48 (m, 2H), 3.19–3.24 (br. m, 1H), 2.29–2.33 (m, 2H), 2.14–2.18 (m, 1H), 2.02 (m, 1H). $^{13}$C NMR (500 MHz, $D_2O$) 185.04, 182.01, 172.42, 167.03, 161.32, 161.30, 156.57, 156.45, 155.51, 153.93, 153.91, 153.64, 131.37, 131.57, 124.13, 124.09, 123.95, 123.89, 114.91, 114.88, 114.74, 114.70, 92.60, 92.57, 45.91, 45.58, 45.56, 44.49, 44.31, 44.21. ESI-MS ($H_2O$/$CH_3OH$) $ESI^+$: $m/z$ 473 ($M + H^+$); $ESI^-$: 471 ($M - H^-$). Anal. Calc. for $C_{20}H_{24}N_8O_6 \cdot 2H_2O$ (508.47): C 47.24, H 5.55, N 22.04; found: C 47.66, H 5.28, N 22.02.

## 3. Results and discussion

The HPLC analysis of CaF, using a C18 column with a phosphate buffer and tetrabutylammonium/MeOH as the mobile phase [4], shows that the product contains less than 0.10% of one apolar impurity (called for convenience impurity **1**; Fig. 2a). The impurity **1,** detected at 280 nm through a diode array detector (DAD), shows a UV–vis spectrum close to the CaF spectrum. This is the first proof that the structure of this impurity is probably similar to CaF (Fig. 2b).

In spite of the fact that the detected level of this impurity in CaF is less than 0.10%, we have decided to characterize this unknown substance in order to increase our knowledge on the process to prepare synthetic CaF.

In general, analysis on LC–MS instruments is done with volatile components in the mobile phase, in order to eliminate contamination the interface between the liquid chromatograph and the mass spectrometer [5]. Therefore, it is necessary to replace the phosphate buffers with volatile components like ammonium bicarbonate and ammonium or triethylamine salts of formic or acetic acid. Moreover, the tetrabutylammonium cation ($TBA^+$) used for HPLC analysis of CaF interferes with the MS analysis because of its strong response and easy fragmentation. After some attempts, a suitable separation method was developed, using a solution

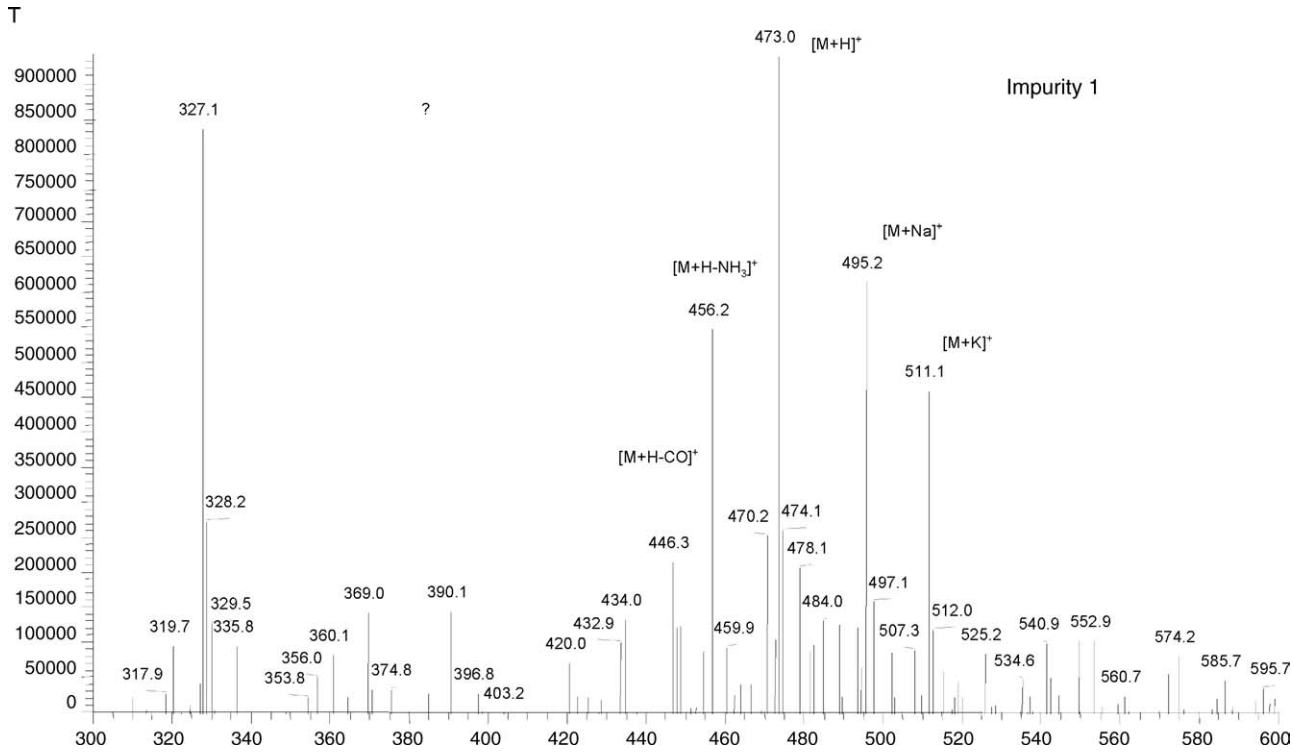



Fig. 3. Positive ion ESI spectrum of the impurity **1**.

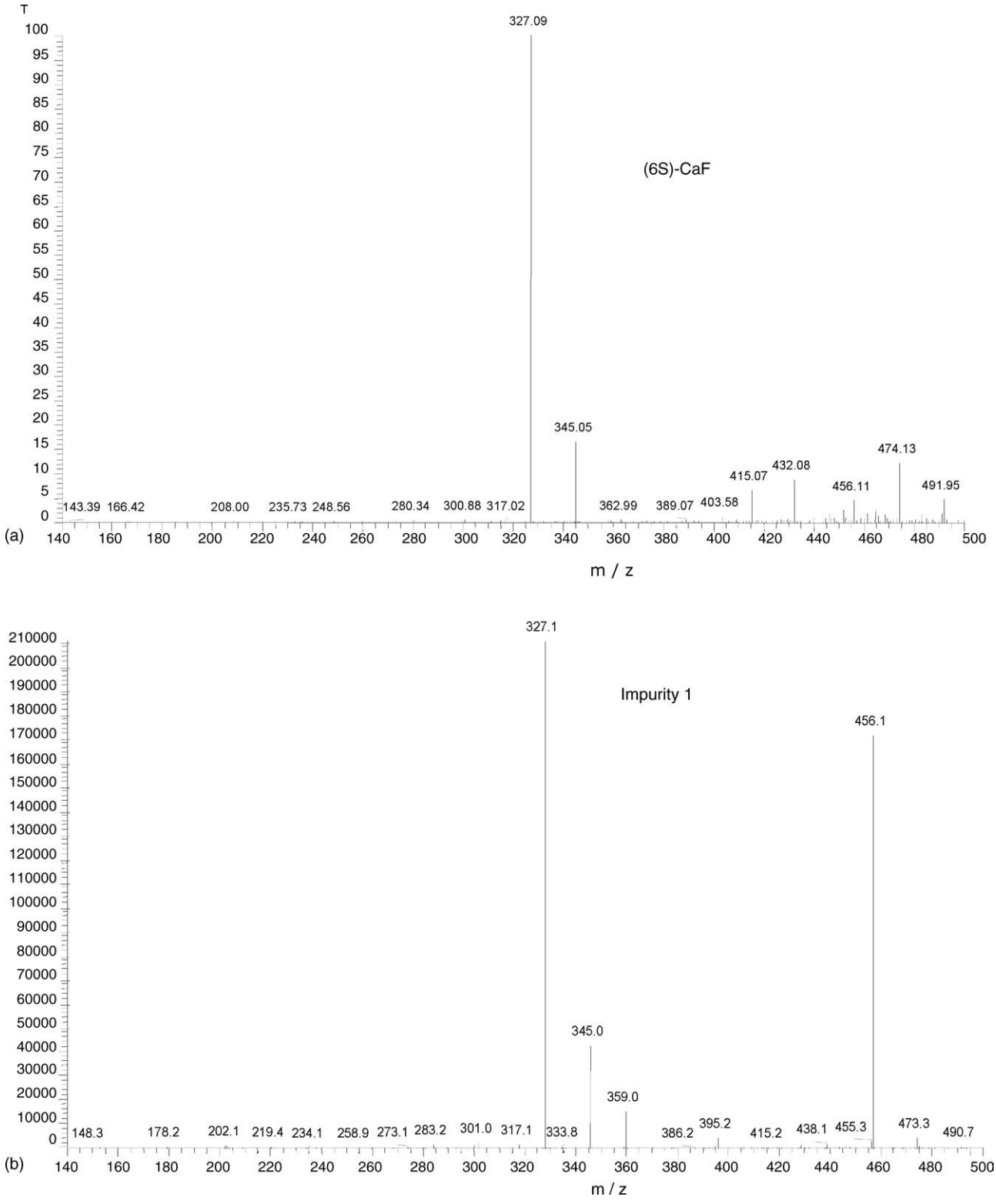



Fig. 4. (a) MS/MS spectrum of CaF obtained through the fragmentation of the ion at *m*/*z* 474. (b) MS/MS spectrum of impurity **1** obtained through the fragmentation at *m*/*z* 473.

Table 1
Fragmentation ions of CaF and impurity **1** (Fig. 4).

| Compound | Postulated ions (*m*/*z*) |
|---|---|
| CaF | 474 $[M+H]^+$ |
| | 456 $[M-H_2O+H]^+$ |
| | 345 $[M-HOOC\text{-}(CH\text{-}NH)\text{-}(CH_2)_2\text{-}COOH+H_2O]^+$ |
| | 327 $[M-HOOC\text{-}CH\text{-}(CH_2)_2\text{-}COOH]^+$ |
| Impurity 1 | 473 $[M+H]^+$ |
| | 456 $[M-NH_3+H]^+$ |
| | 345 $[M-HOOC\text{-}(CH\text{-}NH)\text{-}(CH_2)_2\text{-}CONH_2+H_2O]^+$ |
| | 327 $[M-HOOC\text{-}CH\text{-}(CH_2)_2\text{-}COOH]^+$ |

of ammonium acetate/MeOH as mobile phase, and a $C_{18}$ Hypersil column. The positive ion ESI spectrum of impurity **1** (Fig. 3) showed a protonated molecular ion, $[M+H]^+$ at *m*/*z* 473. There was also another intense peak at 327 *m*/*z* probably due to a fragmentation of the molecule; this was confirmed later on through the MS/MS experiment.

The fact that the value of the molecular weight of impurity **1** is even (472 g/mol) suggests that the number of nitrogen atoms of the unknown substance is also even. To obtain additional structural information, ESI MS/MS analyses were performed both on impurity **1** and CaF.

The MS/MS spectra were obtained through the fragmentation of the ion at *m*/*z* 474 for CaF and at *m*/*z* 473 for impurity **1**. The MS/MS spectra of CaF (Fig. 4a) and impurity **1** (Fig. 4b) contain the same series of fragmentation ions, *m*/*z* 456, 327 and 345 (Table 1), thereby indicating that the two compounds were structurally very similar. The fragmentation product at *m*/*z* 327 is predominant in both MS/MS spectra. This common ion presumably corresponds to the structure shown in Fig. 5, so that CaF and impurity **1** should differ only in the side chain structure: therefore, we postulated that impurity **1** contains a glutaminic moiety (see Fig. 5). A similar fragmentation pathway is already reported in the literature

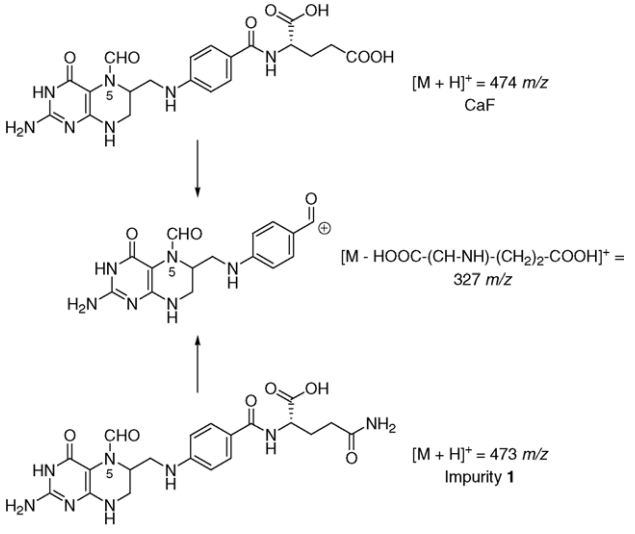
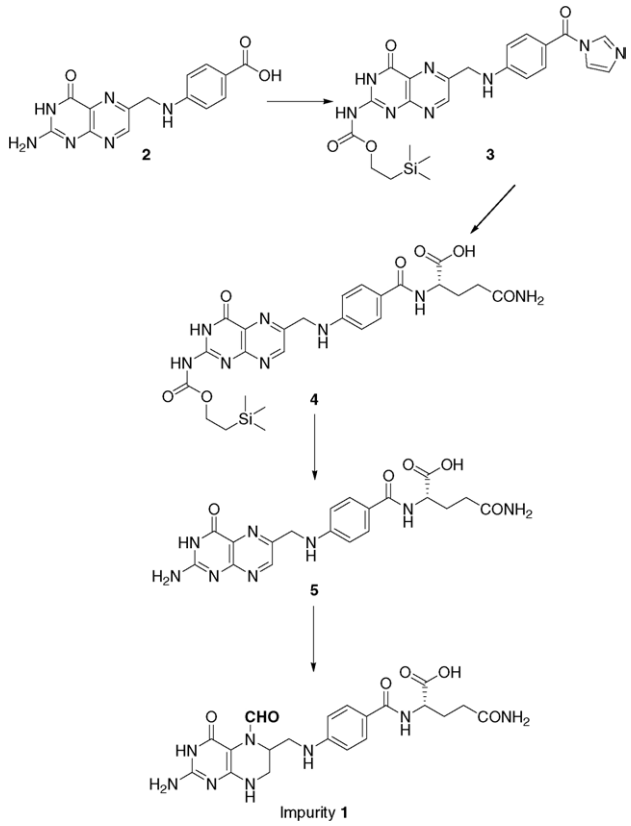



Fig. 5. Fragmentation pathway to give the ion *m*/*z* 327 for CaF and impurity **1**.




Scheme 1. Synthesis of impurity **1**.

for $N^5$-methyl-5,6,7,8-tetrahydrofolic acid, another folic acid metabolite [6].

In order to obtain a definitive proof for the postulated structure of impurity **1** (Fig. 5), a synthetic strategy was developed to prepare this molecule (Scheme 1). Pteroic acid (**2**) was suspended in DMSO and treated with $Et_3N$, 1,1′-carbonyldiimidazole (CDI) and 2-(trimethylsilyl)ethanol to obtain the 1-{2-*N*-[2-(trimethylsilyl)ethoxycarbonyl]pteroyl}-imidazole molecule (**3**), as described in the literature [7] with some modifications. Coupling of **3** with L-glutamine in the presence of *N*-methyl-1,5,9-triazabiciclo[4.4.0]-dec-5-ene (MTBD) gave compuond **4**. This molecule was de-protected by treatment with a solution of tetrabutylammonium fluoride 1 M in tetrahydrofuran to give **5**. Finally, compound **5** was reduced with sodium borohydride and formylated in the presence of formamide to give the impurity **1** [8]. Final proof of the postulated structure of impurity **1**, was obtained by HPLC analysis via co-injection of a CaF sample with the synthesized impurity **1**.

## 4. Conclusions

In order to guarantee the quality and safety of pharmaceutical products it is necessary to develop an efficient analytical approach in order to be able to identify and characterize potential impurities. The LC/MS/MS technique can be used as

a powerful technique for reaching this goal. This work shows an example of the use of LC/MS/MS to identify and then demonstrate, through synthesis, the structure of an impurity contained in our CaF product at a very low level (less than 0.05%). This is very useful information in order to increase the know-how related to our CaF industrial synthesis.

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