Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 1263–1272
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

LC-MS/MS and FT-IR analyses of stones from a patient with Crohn's disease: a case report

Marica Orioli [a], Cristina Marinello [a], Renato Cozzi [b], Luca P. Piodi [c], Marina Carini [a,*]

[a] *Istituto Chimico Farmaceutico Tossicologico, Faculty of Pharmacy, University of Milan, Viale Abruzzi 42, 20131 Milan, Italy*
[b] *Laurea in Tossicologia dell'Ambiente (Lodi)—Faculty of Pharmacy, University of Milan, Milan, Italy*
[c] *Unità Operativa di Gastroenterologia, IRCSS Ospedale Maggiore, Via F. Sforza 35 Milan, Italy*

Received 18 February 2004; received in revised form 18 February 2004; accepted 18 March 2004

Abstract

This report describes the unusual case of a patient affected by Crohn's disease suffering from intestinal obstruction with recurrent occlusive symptoms not due to the intestinal disease, but to the presence of two calcified foreign bodies in the pelvis. The stones were surgically removed and analysed by reverse-phase liquid chromatography coupled to UV diode array detection and mass spectrometry (LC-UV-DAD-MS/MS), Chromatoprobe-MS/MS and by Fourier-transform-infrared spectroscopy (FT-IR) techniques. The combined mass spectrometric approaches allowed unequivocally to identify 5-aminosalicylic acid (5-ASA) in stone 1, and to demonstrate that its formation was due to an unmodified 5-ASA tablet, a formulation that must undergo complete dissolution in the small bowel. The second stone was constituted by a solid layer (no solvent-extractable material) identified by FT-IR as a polystyrene fragment. This indicates that accidental ingestion of a plastic material, followed by its calcification, was responsible for its formation.

Keywords: Crohn's disease; Ileal obstruction; Stones; 5-Aminosalicylic acid; Liquid chromatography tandem mass spectrometry; Polystyrene; Fourier-transform-infrared spectroscopy

1. Introduction: case report

In 1991, a 34-year old male dentist underwent an ileocaecal resection with ileo-colon anastomosis for a stricture of the terminal ileum caused by Crohn's disease. Thereafter, treatment with 5-aminosalicylic acid (5-ASA) tablets was started. The patient remained in good health until spring 2002, when he developed self-limited episodes of diffuse abdominal pain and bloating, suggesting recurrent intestinal occlusion. Being a physician, at first he did not require medical advice and prescribed by himself routine blood laboratory tests, which were within normal limits. Moreover, he underwent a small bowel X-ray follow-through, which did not show evident strictures or dilations of the small intestine. In autumn 2002, after a further occlusive episode, the patient was referred to the Department of Gastroenterology (IRCSS

* Corresponding author. Tel.: +39-02-50317532; fax: +39-02-50317565.
E-mail address: marina.carini@unimi.it (M. Carini).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.03.016

Ospedale Maggiore). At physical examination only a slight tenderness, but no mass was found on right abdominal quadrant and normal peristaltic sounds were heard. He was suggested to perform a plain abdominal X-ray, which showed two calcified foreign bodies in the pelvis. During surgery two intestinal stones placed near to a luminal narrowing of the distal ileum were found and removed, and a stricturoplasty was performed. Diagnosis of Crohn's disease was histologically confirmed on mucosa specimens. The patient recovered and follow-up was uneventful continuing maintenance treatment with 5-ASA. The stones were analysed by electron microscopy which showed an homogeneous central nucleus surrounded by a calcified layer. The particular shape of the stones, as well as their absence in the gall bladder and in bile ducts, suggested that unreleased 5-ASA tablets could be involved in the formation of stones. Aim of this work was to characterise the composition of these stones in order to check the presence of 5-ASA and/or of the coating material used for the gastro-resistant formulation. This was done by a combined approach based on reverse-phase liquid chromatography coupled to UV diode array detection and mass spectrometry (LC-UV-DAD-MS/MS) and on Fourier-transform-infrared spectroscopy (FT-IR) techniques. In parallel we checked the potentiality of the Chromatoprobe DSI (direct sample introduction), a device firstly designed by Amirav and Dagan [1] for sampling of solids and powders and sample introduction into the ion source of a conventional GC-MS system, for rapid monitoring and confirmation of 5-ASA in these biological matrices.

2. Materials and methods

2.1. Chemicals

HPLC-grade and analytical-grade organic solvents were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade water was prepared with a Milli-Q water purification system.

2.2. Stone treatment

Two fragments from the original stone 1 and the intact stone 2 were given to us for analysis. The closely similar fragments a (0.6503 g) and b (0.1254 g) from stone 1 showed a typical shape, which could be indicative of a portion of undissolved tablet, while the thinner stone 2 (0.4878 g) showed the typical shape of a flat kidney-bean. All the fragments were initially analysed in parallel to a commercial 5-ASA tablet (Asalex 60, 400 mg) for the presence of the drug by reverse-phase LC coupled to UV-diode-array detection and electrospray tandem mass spectrometry (ESI-MS/MS). The stones were scraped from the external calcified layer, the 5-ASA tablet from the coloured enteric coating and the cores (tablet and fragments of stone 1) finely powdered. Aliquots (0.1 g) of the powdered materials were extracted twice with 10 ml (tablet) or 4 ml (stone fragments) of 1N HCl and the extracts, after centrifugation at 6000 rpm and filtration through a 0.45 μm filter, directly analysed by LC-MS/MS (the tablet extract was diluted 1:100 with the mobile phase before injection). In parallel, few grains of the powdered fragment a and 5-ASA tablet were submitted as such to chromatoprobe-MS/MS. The internal core of stone 2, a pale and solid layer, was separated into two portions: one was finely minced and treated with HCl for LC-MS/MS analysis as above described, and the second portion was analysed by FT-IR analyses as such and after separation of the surface and internal dissection of a small flake.

2.3. LC-ESI-MS/MS analysis

LC-MS/MS analyses were done on a Thermoquest Surveyor system equipped with a quaternary pump, a Surveyor UV-Vis diode array programmable detector 6000 LP operating at fixed wavelength (295 nm), a Surveyor AS autosampler, a vacuum degasser, a Xcalibur software and connected to a Thermo Finnigan LCQ Advantage Ion Trap Mass spectrometer (ITMS). Separations were done by reverse phase elution with a Sinergy Polar-RP column (150 mm × 2 mm i.d.; particle size 4 μm) protected by a Polar-RP guard-column (4 mm × 2 mm; 4 μm) in the following conditions: gradient elution from 95% solvent A (H_2O:CH_3CN:CH_3COOH, 95:5:0.1, v/v/v) to 15% solvent B (CH_3CN); flow rate 0.2 ml min^{-1}. The ESI/MS source was set as follows: capillary temperature 200 °C; spray voltage 5 kV; capillary voltage 5 V; sheath gas flow rate 2 l min^{-1}; auxiliary gas flow rate 0.5 l min^{-1}. The flow rate of the nebuliser gas

(nitrogen) was 5 l min^{-1}. Spectra were acquired in positive-ion mode, with a scan range from *m/z* 50 to 200 (scan rate 0.5 scans s^{-1}). Collision induced dissociation (CID) experiments in positive-ion mode were performed by selecting the parent ion (*m/z* 154) and the MS^2 product ion (*m/z* 136) with an isolation width of 1 *m/z*, and using helium as collision gas. The ion collision energy was adjusted by changing a percentage of the 5 V a.c. voltage which was applied to the end-caps of the ion trap at the resonance frequency of the selected ion and was set at 30% (referred to as collision energy level).

2.4. Chromatoprobe-MS/MS analysis

MS/MS analyses via direct sample introduction (Chromatoprobe DSI device 1079, Varian, Leinì, Italy) were performed on a conventional GC/MS system equipped with a CP-3800 gas chromatograph and a PTV injector (Varian Instruments, Leinì, Italy) connected to an ion trap mass spectrometer (Saturn 2200, Varian Instruments). To assess the MS/MS resolution power and to shorten the total analysis time, no analytical chromatographic columns were used and the Chromatoprobe, used as an MS probe that efficiently transforms a conventional GC injector into a DSI device, was connected to the ion source via a deactivated short column (DB-1, 2 m × 0.1 mm i.d.; 0.1 mm film thickness) that serves as a fast transfer line. Held by the tip of the Chromatoprobe, a disposable microvial loaded with few grains of the pulverised material (stone or tablet) was inserted into the injector body via the Chromatoprobe guide. Helium was the carrier gas at a head pressure of 15 psi and a flow rate of 1 ml min^{-1} (split 1:30 or 1:5). Starting from 120 °C, the PTV injector was programmed to increase to 300 °C at a rate of 200 °C min^{-1} and then held at 300 °C for 9.1 min. The initial oven temperature was 120 °C for 5 min and then rapidly increased at a rate of 100 °C min^{-1} until 300 °C followed by a 7-min isothermal step. Transfer line, manifold and trap were maintained at 260, 120 and 170 °C, respectively. The ion trap mass spectrometer was operating in EI ionisation (scan mode) in the range 50–200 *m/z* at 1 scan s^{-1}. The parameters used for MS/MS experiments were: parent ion *m/z* 153; CID amplitude 1.2 V; isolation width 3 *m/z*; CID type resonant.

2.5. FT-IR analysis

Fourier-transform infrared spectra were acquired on a Nicolet Nexus FT-IR (Thermo Electron Corporation-Nicolet) equipped with a DTGS detector. An horizontal attenuated total reflectance (HATR) sampling accessory, equipped with a single reflection zinc selenide crystal, was employed for all experiments.

To obtain each spectrum, a background single-beam spectrum of the clean, dry HATR crystal was collected, and immediately after the stone sample was pressed on the crystal for spectrum collection under the same acquisition conditions. Each spectrum represents 32 co-added scans measured at a spectral resolution of 4 cm^{-1} in the 4000–650 cm^{-1} range, at room temperature. The HATR plate was cleaned in situ by scrubbing with isopropyl alcohol and allowed to dry. Spectral data was acquired with Omnic E.S.P.© (enhanced synchronisation protocol) software version 6.1 (Thermo Electron Corporation-Nicolet).

3. Results and discussion

Fig. 1 shows the HPLC-UV-MS profile of the HCl extract from a commercial 5-ASA tablet: the active principle is detectable at RT 2.70 min (a) with a typical UV-DAD fingerprint (λ_{max} 230 and 295 nm) (insert) and a protonated molecular ion $[M + H]^+$ at *m/z* 154. The ESI-MS^3 spectrum (b) obtained by colliding the $[M + H]^+$ and then its fragment at *m/z* 136 $[M + H–H_2O]^+$ unequivocally confirms 5-ASA, with the main product ions at *m/z* 119 $[M + H–H_2O–OH]^+$, *m/z* 108 $[M + H–HCOOH]^+$, *m/z* 92 $[M + H–COOH–H_2O]^+$ and at *m/z* 80 $[108–CO]^+$. The TRC (total reaction chromatogram reported in box c) confirms that all the above reported ions elute with the peak at RT 2.70 min.

HPLC-UV-DAD analysis of the extract relative to fragment a (Fig. 2a) shows a small peak at RT 2.68 min with an UV-DAD profile (insert) consistent with the structure of 5-aminosalicylic acid. MS^3 analysis of the peak unequivocally indicates the presence of the drug, since both the MS/MS fragmentation pattern (Fig. 2b) and the corresponding TRC (Fig. 2c) are super-imposable to those obtained with the tablet. The drug is also present in consistent amount in fragment

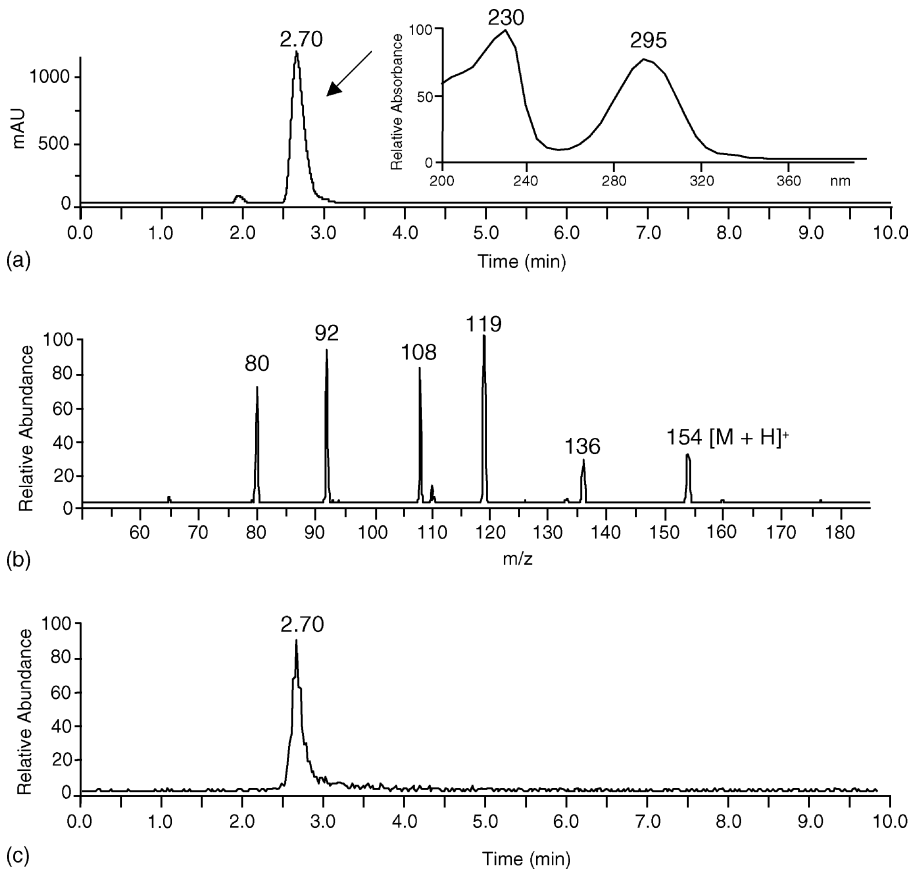

Fig. 1. LC-UV-ESI-MS/MS analysis of the 5-ASA tablet extract. (a) HPLC profile (UV-DAD 295 nm). Insert: UV spectrum of the peak with RT 2.70 min. (b) ESI-MS^3 spectrum of the peak with RT 2.70 min. (c) Total reaction chromatogram (TRC) of the product ions of *m/z* 154.

b (Fig. 2d and e), while is not detectable in stone 2 and no other peaks were found in the UV-DAD and MS profiles (data not shown).

In parallel, to evaluate the potentiality of the Chromatoprobe device for the direct analysis of solid, dirty samples (without any sample preparation) by MS/MS, both the tablet and stone content were analysed in a conventional GC-MS/MS system.

The system was initially calibrated working with the 5-ASA tablet and the results are reported in Fig. 3. The total ion current (TIC) profile (a) indicates the presence of some volatile components (within the first 3 min) and a small peak at 6.83 min, whose EI mass spectrum with the ions at *m/z* 153 $[M]^{+\bullet}$, 135 $[M–H_2O]^{+\bullet}$, 107 $[135–CO]^{+\bullet}$, 79 $[107–CO]^{+\bullet}$, 52 $[79–HCN]^{+\bullet}$, perfectly matches that of 5-ASA found in the library (data not shown). The SIM (single ion monitoring) trace relative to the ion at *m/z* 153 (b) is highly diagnostic and confirms 5-ASA elution around 7 min. For MS/MS experiments, the CID of the ion at *m/z* 153 was performed within the window 5–8 min and the resulting TRC (c) and full scan MS^2 spectrum (d) unequivocally identify 5-ASA. Fig. 4 shows the results relative to fragment a analysed in the MS/MS mode using *m/z* 153 as parent ion. Both the TRC (a) and the full scan MS^2 spectrum (b), identical to those reported for the 5-ASA tablet, confirm the presence of the drug in stone 1.

Stone 2 exhibited the consistency of plastic material and was insoluble both in aqueous and organic solvents (see LC-MS/MS analysis). This behaviour prompted us to investigate its nature by FT-IR, by analysing a fragment as such, after elimination of the external calcified material and its internal core,

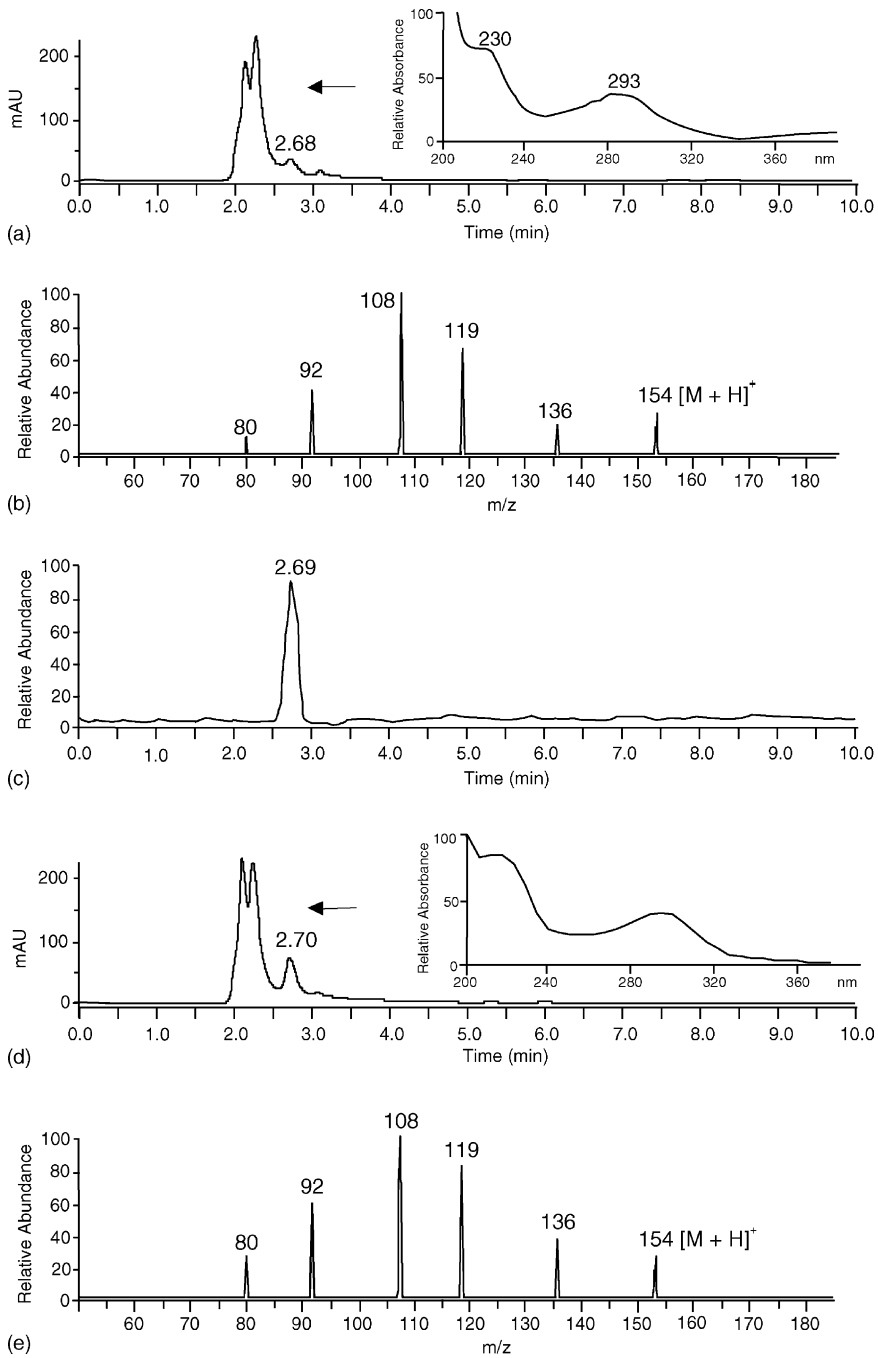

Fig. 2. LC-UV-ESI-MS/MS analysis of the extracts from stone 1. (a) HPLC profile (UV-DAD 295 nm) of fragment a. Insert: UV spectrum of the peak with RT 2.70 min. (b) ESI-MS^3 spectrum of the peak with RT 2.70 min (fragment a). (c) Total reaction chromatogram (TRC) of the product ions of *m/z* 154 (fragment a). (d) HPLC profile (UV-DAD 295 nm) of fragment b. Insert: UV spectrum of the peak with RT 2.70 min. (e) ESI-MS^3 spectrum of the peak with RT 2.70 min (fragment b).

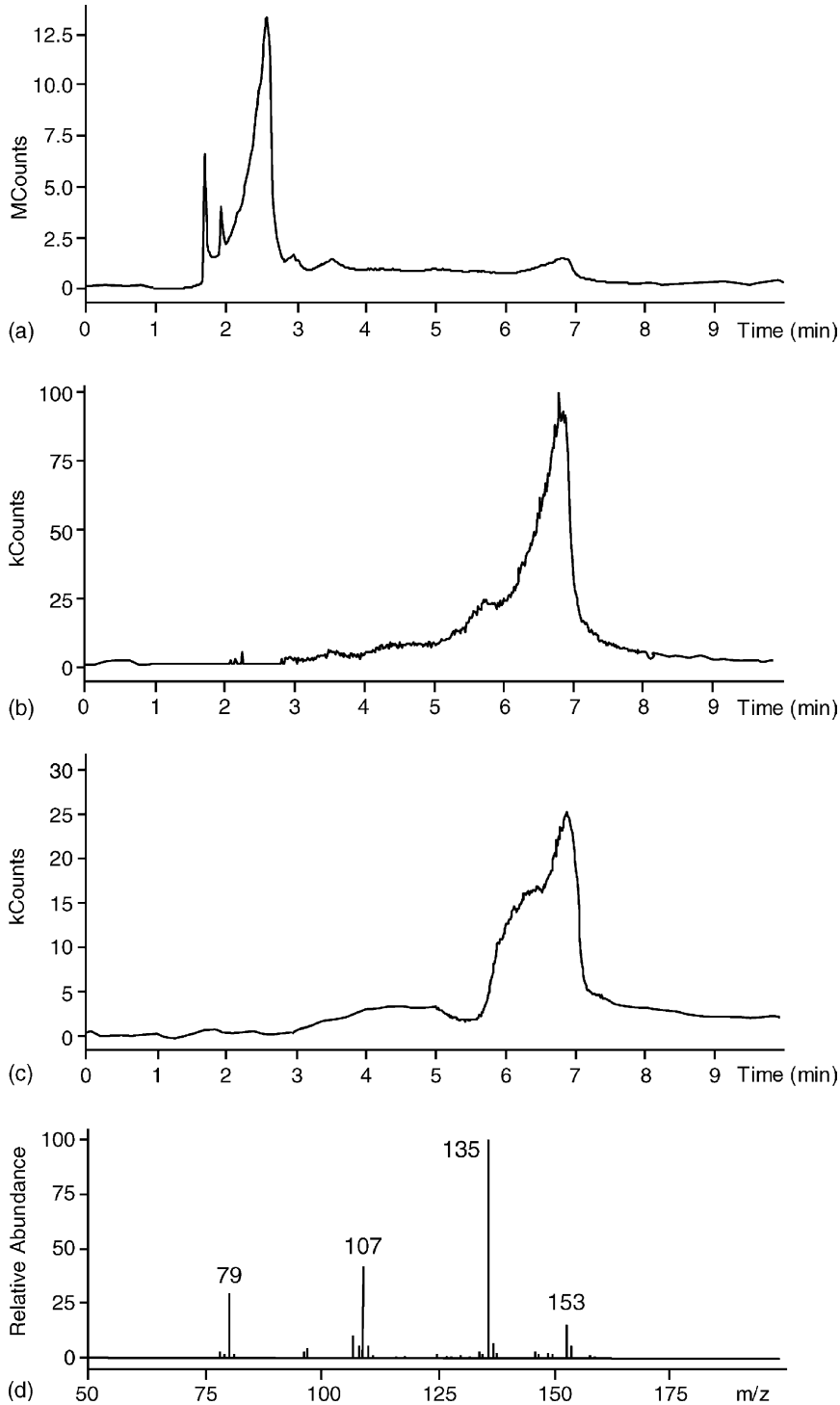

Fig. 3. Chromatoprobe-MS/MS analysis of the 5-ASA tablet (powder). (a) Total ion current (TIC) profile. (b) Single ion monitoring (SIM) trace relative to the ion *m*/*z* 153. (c) Total reaction chromatogram (TRC) of the ion *m*/*z* 153. (d) Full scan MS^2 spectrum of the ion *m*/*z* 153.

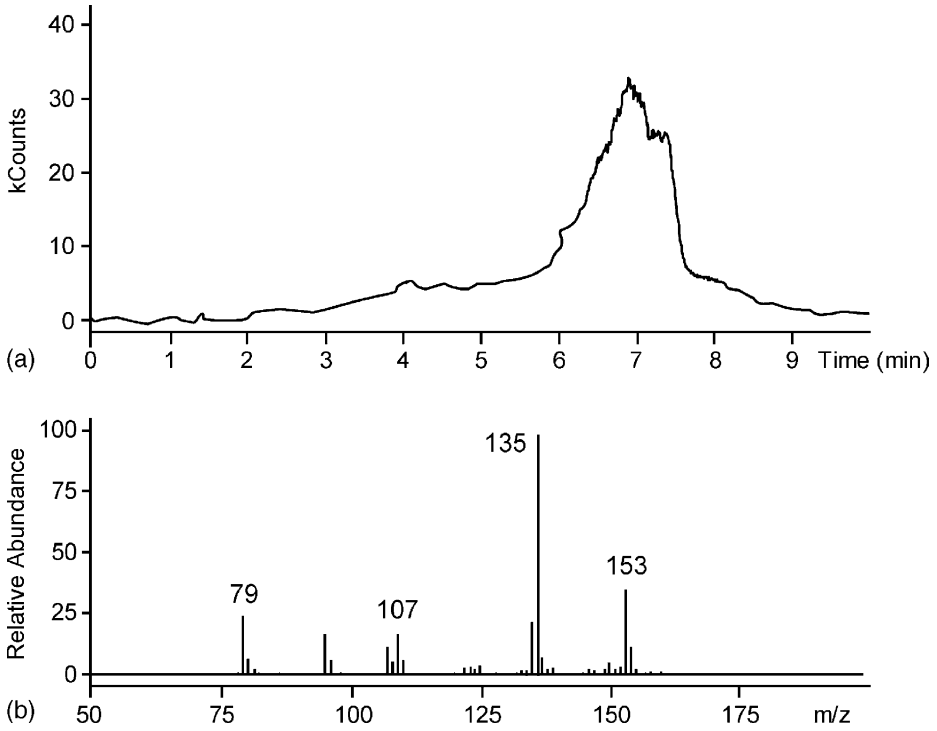

Fig. 4. Chromatoprobe-MS/MS analysis of stone 1 (powder). (a) Total reaction chromatogram (TRC) of the ion *m*/*z* 153. (b) Full scan MS^2 spectrum of the ion *m*/*z* 153.

obtained by careful dissection of a small flake. The FT-IR spectra of the fragment and of the internal core (Fig. 5a) show diagnostic bands for different functional groups that allowed to characterise the main sample components. In detail, the following more interesting bands:

Broad band centered at 3274 cm^{-1}	O–H and N–H stretch
3100–3010 cm^{-1}:	=C–H stretch (aromatic ring)
3000–2800 cm^{-1}	C–H aliphatic stretch
1950–1730 cm^{-1}	Overtone/combination bands of aromatic compounds
1650–1620 cm^{-1}	Amide I band
1600, 1492 and 1451 cm^{-1}	C=C stretch aromatic ring
1541 cm^{-1}	Amide II band
1241 cm^{-1}	Amide III band
Broad band centered at ~1100 cm^{-1}	C–O stretch
1073 and 1028 cm^{-1}	In plane =C–H bending
747 and 692 cm^{-1}	Out of plane =C–H bending (mono-substituted aromatic ring)

clearly indicate mono-substituted benzene rings together with amide and alcohol functions, these last probably due to incomplete removal of the calcified external layer (endogenous organic matter). In fact, the difference spectrum recorded in the more interesting region (Fig. 5b), point out the presence of protein material on the external surface of stone 2 and indirectly confirm the aromatic component in the internal layer. Definitive assignment was achieved by comparing its FT-IR spectrum with that of a polystyrene

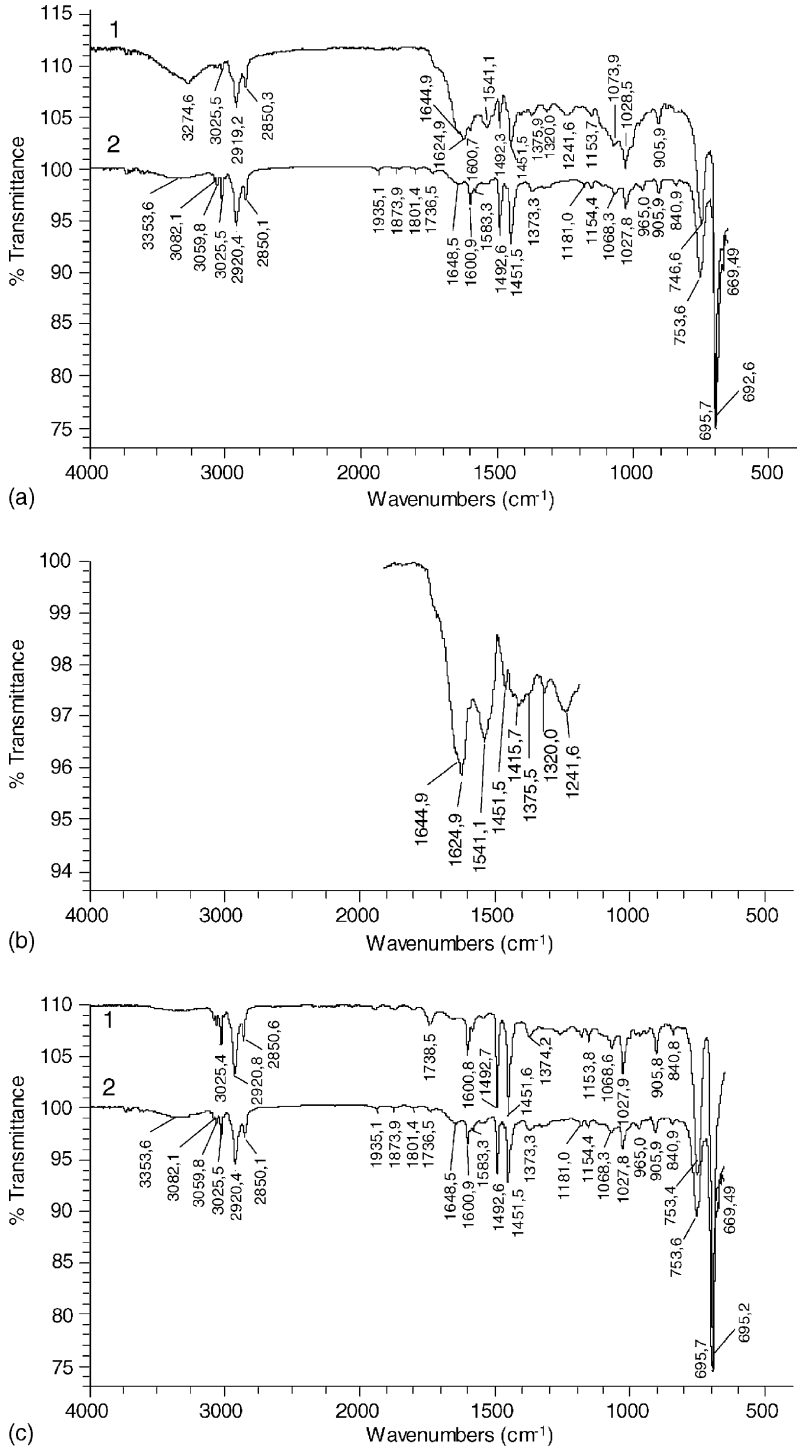

Fig. 5. FT-IR analysis of stone 2. (a) Whole fragment (1) and internal layer (2). (b) Difference spectrum (subtraction of spectrum 2 from spectrum 1). (c) Polystyrene standard (1) and internal layer (2).

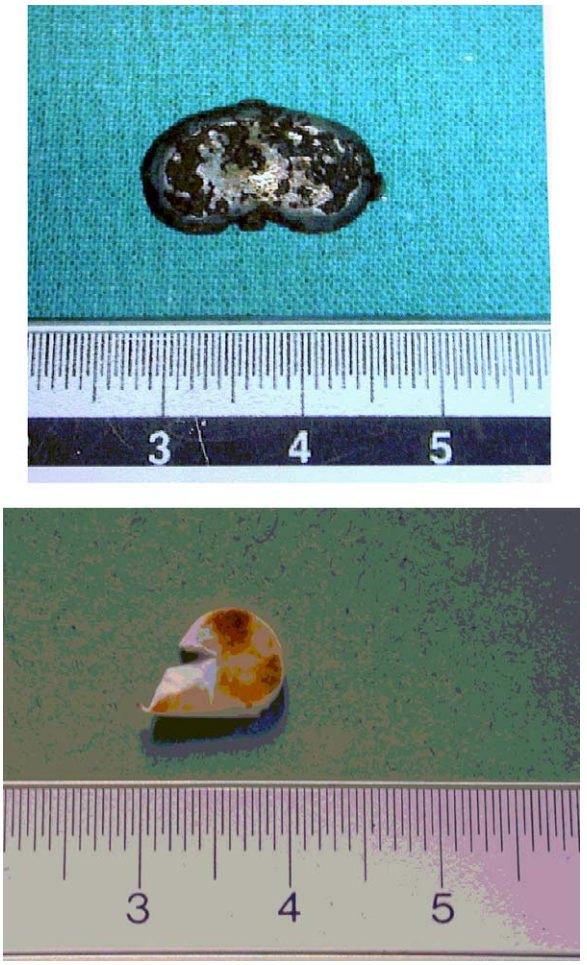

Fig. 6. Intact stone 2 (top) and a portion of its internal core (bottom).

standard (Fig. 5c) and the perfect match between the two spectra allows to unequivocally identify the nature of the stone. Hence the shape, the typical consistency (plastic material) and the FT-IR fingerprint of the internal layer of stone 2 indicate that formation of the smaller stone might be due to accidental ingestion of a polystyrene fragment (for example from a small plastic spoon). Fig. 6 shows the original stone 2 and a portion of its internal layer.

4. Conclusions

The results of this study, based on the use of two largely different mass spectrometric approaches (ESI-MS/MS and EI-MS/MS) unequivocally confirm the presence of 5-ASA in the two fragments of the intestinal stone 1 surgically removed from the distal ileum of the patient. From a clinical and toxicological point of view, these results are of relevant interest. No cases of intestinal obstruction caused by 5-ASA tablets have been until now reported, taking into account that currently used formulations must undergo complete dissolution in the small bowel. Hence, this represents an unusual case of a patient affected by Crohn's disease suffering from intestinal obstruction with recurrent occlusive symptoms not due to the intestinal disease itself, but to an undissolved 5-ASA tablet that, stopping at the ileal narrowing, caused subocclusive symptoms with a valve-like mechanism. Even more interesting are the results relative to the FT-IR analysis of the smaller stone constituted by polystyrene, because accidental ingestion of a plastic fragment represents another unusual case of stone formation and the finding here reported represents to our knowledge the first example.

From an analytical point of view, the results of this study from one side extend the well known versatility of the FT-IR technique (until now never applied in this specific bio-analytical field), from the other confirm the usefulness of liquid chromatography coupled to UV-DAD detection and electrospray mass spectrometry in the tandem mode for a rapid and complete on-line identification (unequivocal molecular weight and structure assignment) of a target compound in a complex biological matrix. Concomitantly they indicate new fields of application for the Chromatoprobe device coupled to a GC/MS system in analytical toxicology. Until now the Chromatoprobe, allowing large volumes injection, has been mainly used as an introduction device for conventional capillary GC-MS or GC-MS/MS analysis of target compounds in dirty samples such as human urine [1], brain [2] and hair [3], food items such as blended fruits, vegetables and spices [4–7], but no applications are reported in literature for direct MS/MS analysis of solid samples, as described in this work.

The mass spectrometer, operating in the MS/MS mode, eliminates the background interferences from the solid matrix and detects only the analyte of interest, allowing to avoid separation by an analytical GC column. The main advantage of Chromatoprobe is

that qualitative MS spectral information can be generated on solid samples without any sample manipulation. Unlike traditional MS probes that are inserted into the ionisation source, the Chromatoprobe is designed to function in a temperature-programmable injector body and sample delivery to the MS can be adjusted either by injector temperature or split flow ratios, avoiding problems due to overloaded MS source; in addition non-volatile or thermally degraded components from the matrix remain in the Chromatoprobe vial and can never contaminate the ion source.

References

[1] A. Amirav, S. Dagan, Eur. J. Mass Spectrom. 3 (1997) 105–111.
[2] R. Carpenedo, V. Carla, G. Moneti, A. Chiarugi, F. Moroni, Anal. Biochem. 244 (1997) 74–79.
[3] S.B. Wainhaus, N. Tzanani, S. Dagan, M.L. Miller, A. Amirav, J. Am. Soc. Mass Spectrom. 9 (1998) 1311–1320.
[4] H. Jing, A. Amirav, Anal. Chem. 69 (1997) 1426–1435.
[5] A. Amirav, J. Hing, J. Chromatogr. A 814 (1998) 133–150.
[6] S.J. Lehotay, J. AOAC Int. 83 (2000) 680–697.
[7] M. Kochman, A. Gordin, P. Goldshlag, S.J. Lehotay, A. Amirav, J. Chromatogr. A 974 (2002) 185–212.