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High-performance liquid chromatography coupled to ion spray mass spectrometry for the determination of colchicine at ppb levels in human biofluids

A. Tracqui*, P. Kintz, B. Ludes, C. Rougé, H. Douibi, P. Mangin

Institut de Médecine Légale, 11 rue Humann, 67085 Strasbourg, France

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Abstract

An original method based upon high-performance liquid chromatography coupled to ion spray mass spectrometry (HPLC–ISP-MS) has been developed for the identification and quantification of colchicine (COL) in human blood, plasma or urine. After single-step liquid-liquid extraction by dichloromethane at pH 8.0 using tofisopam (TOF) as an internal standard, solutes are separated on a 5-μm C_{18} Microbore (Alltech) column (250×1.0 mm, I.D.), using acetonitrile–2 mM NH_4COOH, pH 3 buffer (75: 25, v/v) as the mobile phase (flow-rate 50 μl/min). Detection is done by a Perkin-Elmer Sciex API-100 mass analyzer equipped with a ISP interface (nebulizing and curtain gas: N_2, quality U; main settings: ISP, +4.0 kV; OR, +50 V; Q0, −10 V; Q1, −13 V; electron multiplier, +2.2 kV); MS data are collected as either total ion current (TIC, *m/z* 100–500 or 380–405), or selected ion monitoring (SIM) at *m/z* 400 and 383 for COL and TOF, respectively. COL mass spectrum shows a prominent molecular ion $[M+H]^+$ at *m/z* 400. Increasing OR potential fails to provide a significant fragmentation. Retention times are 2.70 and 4.53 min for COL and TOF, respectively. The quantification method shows a good linearity (r=0.998) over a concentration range from 5 to 200 ng/ml. The lower limit of detection in SIM mode is 0.6 ng/ml COL, making the method convenient for both clinical and forensic purposes.

Keywords: Liquid chromatography–mass spectrometry; Colchicine

1. Introduction

Colchicine (COL; Fig. 1) is a naturally occurring alkaloid, obtained from the corm and seeds of the autumn crocus (=meadow saffron, *Colchicum automnale*) and other Liliaceae such as the glory lily (*Gloriosa superba*). It has been used since 600 A.D. for the relief of joint pain, and is presently the drug of choice in acute gouty arthritis [1–4]. It has been also reported to be effective for the treatment of familial Mediterranean fever and some skin diseases, and more recently has been proposed as an experimental antineoplastic agent [2–5]. COL is an antimitotic drug and both its therapeutic and toxic

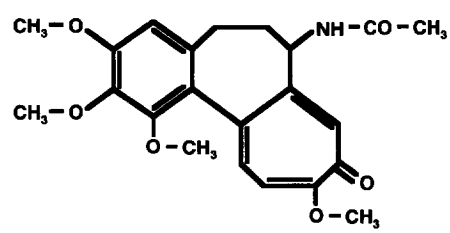

Fig. 1. Chemical structure of COL.

*Corresponding author

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activity are mainly related to its ability to inhibit microtubule polymerization. COL poisonings are rare but always severe. Clinical features are well documented and comprise early complications (gastrointestinal symptoms, volume depletion, peripheral leukocytosis) followed by a multisystem organ failure involving cardiorespiratory, nervous, renal and haematologic systems; ingested doses >0.8 mg/kg are considered to be invariably fatal whatever the supportive care employed [5–12].

A number of methods have been developed for the determination of COL in biological fluids, namely colorimetry [13–16], radioisotope dilution [17,18], radioimmunoassay [19–21], indirect atomic absorption spectrometry [22], fluorimetry [23], GC–MS [5] as well as HPLC with either single-wavelength UV [9,24–27] or diode-array detection [12]. We present in this paper the first procedure using HPLC coupled with ion spray mass spectrometry (HPLC–ISP-MS) for convenient identification and quantification of COL in human biofluids at ppb (ng/ml) levels.

2. Experimental

2.1. Materials

COL (free base, MW=399.43) and the internal standard (I.S.) tofisopam (TOF) (free base, MW= 382.46) were obtained from Sigma-Aldrich (St-Quentin-Fallavier, France) and Biogalénique (Paris, France), respectively. Methanol, acetonitrile and dichloromethane were HPLC grade (Merck, Darmstadt, Germany). Concentrated (99–100%) formic acid (HCOOH) was Normatom grade (Prolabo, Paris, France). Dibasic ammonium phosphate ($(NH_4)_2HPO_4$), concentrated (ca. 85%) orthophosphoric acid (H_3PO_4), and ammonium formate (NH_4COOH) were analytical grade and purchased from Prolabo (Paris, France) and Fluka (St-Quentin-Fallavier, France).

Stock solutions of COL and TOF were prepared in methanol at a concentration of 100.0 μg/ml and stored at +4°C in the dark, where they were found to be stable for at least one month. Due to the light-sensitivity of COL [1–3,23,27], work solutions were prepared just before use by appropriate methanolic dilutions in 1.5-ml Eppendorff-type plastic microtubes made opaque using aluminium foils.

The different buffers were prepared with bidistilled water, deionized before use by passing it through a reverse-osmosis four-filter purification system (Milli-Q, Millipore, Bedford, MA, USA). The pH 8 buffer was prepared using a 1 *M* $(NH_4)_2HPO_4$ solution (132.1 mg/ml), adjusted to the desired pH by appropriate addition of conc. H_3PO_4. Mobile phase buffer optimization was carried out using a 2 m*M* NH_4COOH solution (126.2 μg/ml), adjusted at several pHs (2.0, 2.5, 3.0, 4.0, 5.0, 5.7) by appropriate addition of conc. HCOOH.

A 10^{-4} *M* solution of high-MW polypropylene glycols (PPGs) in water–methanol (50:50, v/v +2 m*M* ammonium acetate +0.1% formic acid +0.1% acetonitrile), provided by Perkin-Elmer Sciex (Foster City, CA, USA) was used for mass analyzer tuning.

2.2. Chromatography

A 20-ml dual-syringe HPLC pump (Applied Biosystems Model 140B, Foster City, CA, USA) was employed to deliver the pulse-free, low flow-rates required by the ISP interface. Samples were manually injected using a Hamilton Model 1710 (Reno, NV, USA) 100-μl gastight syringe and a Rheodyne Model 8125 (Cotati, CA, USA) low-dispersion valve equipped with a home-made, 0.6-μl PEEK loop (0.0025 in. I.D.). Applications entailing continuous infusion of a definite analyte (e.g. MS tuning or spectrum determinations) were carried out using a precision, single-syringe low-pressure infusion pump (Harvard Apparatus Model 11, South Natick, MA, USA) and a 1-ml gastight syringe (Hamilton Model 1001).

The HPLC separations were performed on a 5-μm C_{18} Microbore (Alltech, Deerfield, IL, USA) column (250×1.0 mm I.D.), operated at ambient temperature and protected by a 5-μm C_{18} MGU-80 (LC Packings, Zürich, Switzerland) micro-guard column (1.0×0.8 mm I.D.). The elution was achieved isocratically (flow-rate 50 μl/min, average operating pressure 8.8 MPa) with a mobile phase of acetonitrile–2 m*M* NH_4COOH/pH 3.0 buffer (75:25, v/v) before use, this mobile phase was degassed and filtered through 0.45-μm filters (Durapore GVWP 047, Millipore, Bedford, MA, USA)

with a Pyrex filter holder (Millipore). Due to the microbore column, an equilibration time of at least 3 h was necessary before performing analyses; at the end of each chromatographic session, the column was thoroughly rinsed with a mixture of acetonitrile – deionized water (50: 50, v/v) at a flow-rate of 50 μl/min for 3 h.

The different components of the chromatographic system were connected using 0.005 in. I.D. PEEK tubing and Fingertight Model F300 PEEK fittings (Upchurch Scientific, Oak Harbor, WA, USA), except for the transfer line to the ISP where a 7-cm, 0.0025 in. I.D. PEEK tubing (inner volume ca. 0.22 μl) was used to minimize the post-column dead volumes.

2.3. Mass spectrometry

MS detection was carried out using a Perkin-Elmer Sciex (Foster City, CA, USA) API-100 double-quadrupole instrument. The system was monitored by an Apple Macintosh PowerPC 8100/80 computer equipped with the softwares LC2Tune v. 1.1, MultiView v. 1.1, and MacQuan v. 1.4 (Perkin-Elmer Sciex) for instrument control and data acquisition, data reprocessing, and solute quantification, respectively.

Nitrogen (purity grade U, i.e. 99.95%, L'Air Liquide, Paris, France) was employed as the nebulizing gas at a pressure of 40 p.s.i. (flow-rate 1.16 l/min). The instrument was operated in the positive ionization mode with a voltage of +4.0 kV applied to the sprayer during all experiments. Ions generated in the ion source were sampled into the mass analyzer by passing through a 25-μm I.D. orifice (OR, defined by a variable voltage from 0 to +200 V) at the rear end of the atmospheric chamber. To prevent solvent vapours and contaminants from entering the vacuum chamber, the area in front of the OR was continuously flushed with a 'curtain gas' (N_2, purity U, from L'Air Liquide, operating pressure 40 p.s.i.) at a flow-rate of 1.08 l/min during all experiments, and 0.14 l/min when the instrument was set in overnight standby.

The system was tuned weekly by using a continuous infusion at 5 μl/min of the standard mixture of PPGs, and monitoring the ions at *m/z* 59, 175, 616, 907, 1255, 1545, 1836 and 2010 for mass calibration, lens optimization and peak width adjustments. For routine COL determinations, the main instrument settings were: OR, +50 V; Q0, −10 V; IQ1 (lens), −12 V; ST (lens), −15 V; Q1, −13 V; EM, +2200 V. MS data for COL and the I.S. TOF were collected using either total ion current (TIC) with a range of *m/z* 100–500, then 380–405 (step size 0.2 a.m.u., dwell time 20 ms, scan time 2.5 s) after it has been established that COL provided no interesting low-mass fragments (see results), or in the selected ion monitoring (SIM) mode at *m/z* 400±0.5 and 383±0.5 (step size 0.2 a.m.u., dwell time 250 ms, scan time 3.0 s) for COL and TOF, respectively.

2.4. Extraction procedure

To 4.0 ml of blood, plasma or urine in 15-ml Pyrex centrifuge tubes were added 20 μl of a 1.0-μg/ml methanolic solution of TOF, 1.5 ml of the $(NH_4)_2HPO_4$ buffer (pH 8.0), and 4.5 ml of dichloromethane. This mixture was gently shaken on a horizontal agitator for 10 min, then centrifuged at 3500 *g* for 10 min. The lower organic phase was removed into 5-ml borosilicate glass tubes and evaporated at 45°C in a rotary evaporator (Speed Vac Concentrator Model A 290, Savant Instruments, Hicksville, NY, USA) until reduction of its volume to ca. 1.0 ml; it was then transferred into 1.5-ml Eppendorff-type plastic microtubes and evaporation was completed to dryness. After adding 30 μl of the mobile phase, vortexing (10 s) and centrifuging (10 000 *g* for 5 min), 20 μl of the supernatant were pipetted, from which 0.6 μl were injected onto the column at each chromatographic run.

3. Results and discussion

Fig. 2. presents the full-scan (*m/z* 100–500), background-subtracted positive-ion ISP mass spectrum of COL (OR=+30 V). This was recorded from a continuous, 5 μl/min syringe infusion of a 1.0 μg/ml solution of the analyte, obtained by decimal dilution of a 10.0 μg/ml methanolic solution of COL with the 2 m*M*, NH_4COOH buffer (pH 3.0). The spectrum is quite simple, showing a major peak at *m/z* 400 due to the protonated COL $[M+H]^+$, small peaks at *m/z* 422 and 438 corresponding to sodium and potassium clusters $[M+Na]^+$ and [M+

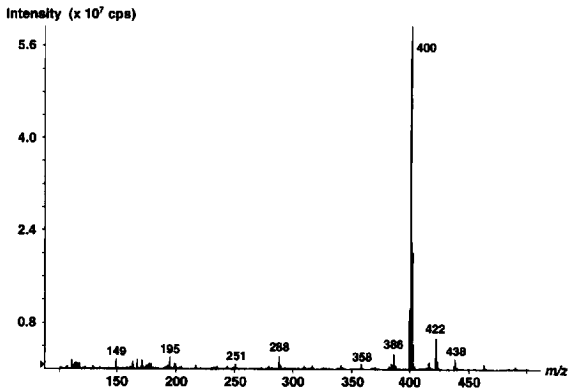

Fig. 2. Positive-ion, ISP mass spectrum (m/z 100–500) of COL. Conditions: Infusion (5 μl/min) of a 1.0 μg/ml solution of COL in methanol–2 mM NH_4COOH, pH 3.0 (10:90, v/v); OR=+30 V.

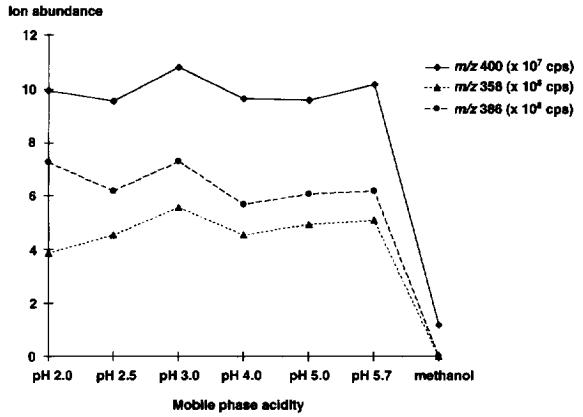

Fig. 3. Influence of mobile phase acidity on COL ionization. Conditions: Infusion (5 μl/min) of a 10.0 μg/ml solution of COL in methanol–2 mM NH_4COOH, pH 3.0 (10:90, v/v); OR=+30 V; SIM recording at m/z 400, 386 and 358; each datapoint is the mean of six successive measurements.

$K]^+$ (inconstantly observed depending upon the matrix composition), and some constant, low-abundance peaks at m/z 386, 370 and 358, representing putative COL fragments. For such spectrum determinations under positive-ion conditions, it is important to inject buffered, acidic solutions of the analyte instead of pure organic solutions to ensure a good protonation of the molecule; in the present case, the use of a pure, 1.0-μg/ml methanolic solution of COL led to a dramatically reduced intensity of the TIC (thus an important loss in sensitivity), the sodiated cluster $[M+Na]^+$ becoming the major ion due to the lack of protons. Nevertheless, the optimization of the pH of the mobile phase does not appear to be so important (provided it is acidic), since we could not observe significant variations in the abundance of COL-related ions over the pH range 5.7 to 2.0 (Fig. 3).

The preferential formation of the molecular ion and the low abundance or absence of fragment ions is a common character of mass spectra generated by the different atmospheric pressure ionization (API) interfaces [electrospray (ESP), ISP, as well as atmospheric pressure chemical ionization, APCI], that involve much less energetic processes than the classical electron impact (EI) ionization mode [28–30]. However, it has been previously reported for various analytes that increasing the potential in the region of the ion-sampling OR may increase the energy of local ion-molecule collisions, resulting in fragmentation of molecular ion species via collision-induced dissociation (CID)-like mechanisms – a so-called 'poor man's MS–MS' [28,29,31,32]; this may provide some structural information and a substantial enhancement of the mass spectrum specificity. Unfortunately this was not the case for COL: SIM recording of the molecular ion (m/z 400) and two of its putative fragments at m/z 386 and 358 while increasing the OR voltage from +10 to +200 V (infusion conditions as above) showed for the three ions an abundance optimum in the region +40 to +70 V, but full-scan monitoring at higher potentials failed to reveal the formation of characteristic low-mass ions; this probably results from immediate dissociation of such ions into smaller fragments, combined with the formation of multiply-charged ions (a typical feature of both ESP and ISP processes), as suggested by the periodic distribution of masses observed [29,33–35].

Flow injection analysis (FIA) experiments were then carried out in order to determine the absolute sensitivity of the mass detector when assaying pure COL solutions. The injection valve was directly connected to the mass analyzer inlet using a 40-cm, 0.0025 in. I.D. PEEK tubing (inner volume ca. 1.27 μl); mobile phase composition and flow-rate were as mentioned above. SIM was done at m/z 400±0.5 while running series of methanolic dilutions prepared from the 100.0-μg/ml COL stock solution. A 0.6-μl

injection was performed for each concentration. The absolute limit of detection (LOD), defined as a signal-to-noise ratio of 3, was reached with a 7.0 ng/ml dilution, i.e. 4.2 pg COL injected.

This exceptional sensitivity must not conceal that FIA cannot be a well-suited technique for quantitative analysis of extracts from complex biological matrices, due to the unavoidable co-extraction of many endo- or exogenous compounds that may interfere by competition during the ionization process, or by possible coincidence of their MW with that of the analyte. On these grounds we have subsequently developed a HPLC procedure for the determination of COL in human biofluids. A single-step liquid-liquid extraction using dichloromethane was employed for reasons of speed and convenience, as usual chlorinated solvents have widely proven to be the media of choice for COL extraction [5,9,12,23–27]. Absolute recovery was determined by extracting and assaying in the SIM mode blank plasma samples loaded with COL at concentrations of 10.0 and 50.0 ng/ml, and by comparing the representative peak areas of these extracted samples with those of unextracted methanolic standards at the same concentrations; results (expressed as mean±S.D. of six separate experiments/concentration) were 86.2±4.3% and 90.8±4.5%, respectively. The HPLC separation was performed on a microbore-type column, as such devices are well adapted to the low flow-rates imposed by the ISP technique, thus require no post-column splitting. Under the chromatographic conditions described here, average retention times (t_R) for COL and TOF were 2.70 and 4.53 min, respectively (corresponding k' values vs. uracil: 0.29 and 1.16), ensuring both a short analysis time and an adequate resolution between analyte and I.S.

The search for an appropriate I.S. raised some difficulties: COL analogues such as colchiceine, desmethylcolchicine, N-methyl-N-desacetylcolchicine (demecolcine or Colcemid, Ciba, Basel, Switzerland), desacetylthiocolchicine, trimethylcolchicineic acid as well as the different colchicosides obviously cannot be employed since they are naturally present as congener alkaloids in *C. automnale* preparations and/or appear as steps of COL metabolic pathways [1–3,9,23,36]. Deuterated standards of COL or analogues are not available on the market. Some authors employed quinidine as an I.S., since both this drug and COL exhibit marked absorbance in the UV region 330 to 350 nm [25,27]. An original solution was proposed by Lhermitte et al. [26] by using morpholinopropylcolchicamide, a home-made, synthetic analogue of COL, however this latter technique is tedious and entails the operator to be experienced in organic synthesis. Finally we chose TOF for I.S.: Although not structurally related to COL, TOF presents a close M_r (which allows the use of a narrow TIC at the early step of the analysis, resulting in improved sensitivity), is well co-extracted (recovery >90% at the concentration used) and elutes with good resolution towards COL; in addition this, anxiolytic benzodiazepine (Sériel®, Biogalénique Labs.) is of very unusual prescription in France. Alternatively, quinacrine (M_r=399.96; an antimalarial not marketed in France) or alpidem (M_r=404.34; an anxiolytic removed in 1994 from the French market) may be employed since they were found to satisfy the same requirements.

COL quantifications were realized by computing peak area ratios (COL/TOF) of the sample extracts analyzed in SIM mode, and comparing them with the calibration curve; this six-point standard curve was constructed by assaying drug-free plasma samples spiked to contain COL at concentrations of 0, 5.0, 10.0, 20.0, 50.0 and 200.0 ng/ml (each analysis performed in duplicate) (Fig. 4). Results showed a good linearity (r=0.998) over the concentration range tested, with an equation of $y=34.223x-0.672$ (y=COL concentration in ng/ml; x=COL area/TOF area).

Accuracy and precision for the assay were determined by extracting and assaying aliquots of pure plasma fortified with COL at 10.0 and 50.0 ng/ml (10 replicates for each level). The measured concentrations (mean±S.D.) were 9.78±1.06 ng/ml and 51.17±4.67 ng/ml, respectively (accuracy and precision: 2.2% and 10.8% at 10 ng/ml, 2.3% and 9.1% at 50 ng/ml). The day-to-day precision, estimated by daily analysis of an aliquot of plasma loaded with COL at 50.0 ng/ml over a period of 10 days, was 12.6%; this result was found acceptable, however we recommend the performance of a new calibration at the beginning of each chromatographic session.

Sensitivity is a major criterion for every method devoted to the analysis of COL, since this compound

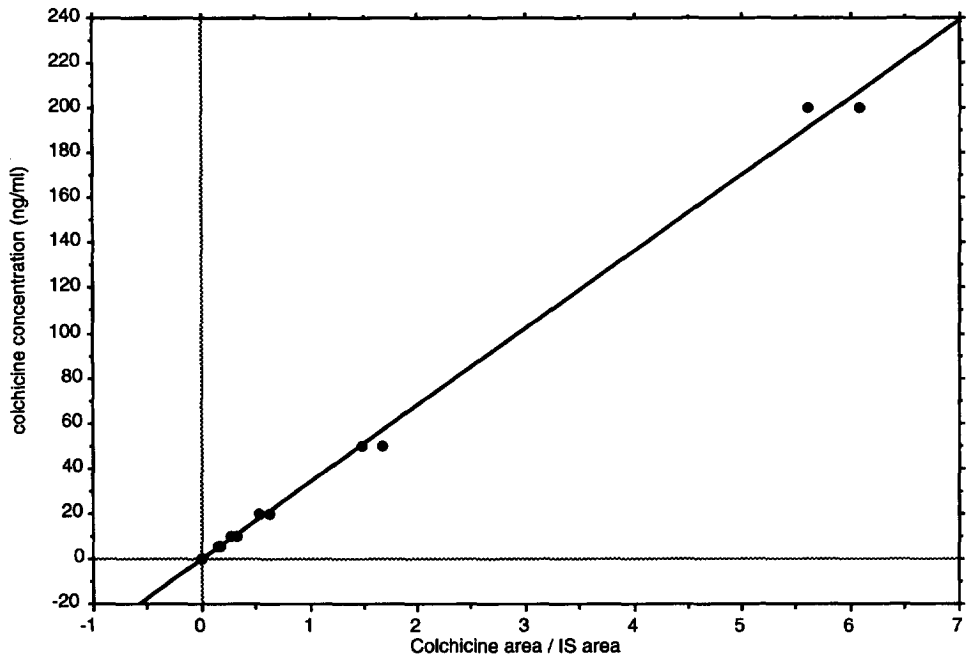

Fig. 4. Calibration curve for COL quantification using TOF as the internal standard. Data recorded in SIM mode at m/z 400 and 383. Column: 5-μm C_{18} Microbore (Alltech), 250×1.0 mm I.D. Eluent: acetonitrile–2 m*M* NH_4COOH, pH 3 (75:25, v/v); flow-rate 50 μl/min. $y=34.223x-0.672$; $r^2=0.997$.

is therapeutic and toxic at particularly low doses. Therapeutic plasma concentrations have been reported to be 3.23±1.73 ng/ml (C_{max} after 1-mg single oral administration to ten subjects) [37], or in the range 0.3 to 2.4 ng/ml at steady-state (1 mg daily to eight subjects) [38]. COL measurements in fatalities have been only seldom performed, showing blood or plasma levels in the range 10–250 ng/ml [6,12,24,25,39]; individual concentrations are difficult to compare and strongly depend upon the timepoint of blood sampling, due to the extremly short plasma half-life of COL (ca. 20 min) contrasting with the fact that poisoned persons frequently undergo a prolonged agony. Our lower limit of detection (determined by extracting and assaying in SIM mode pure plasma or urine samples spiked with decreasing concentrations of COL until a response equivalent to three times the background noise was obtained) was about 0.6 ng/ml for both matrices. This good sensitivity makes our method particularly convenient for COL screening and quantification in human fluids in case of suspected poisoning, in clinical as well as forensic situations. As an example, Fig. 5(a) presents the TIC (m/z 380–405) chromatogram of a 1-ml blood sample from a 24-year-old male who died in the intensive care unit under circumstances suggesting a COL overdosage. Fig. 5(b) shows the extracted chromatogram (obtained by reprocessing the TIC and focusing upon the sole masses of interest, *i.e.* 383 and 400), which illustrates the dramatic improvement of the signal-to-noise ratio gained in that way. The measured blood concentration was 96 ng/ml.

4. Conclusion

The present method is the first described for the analysis of COL in biological fluids by means of HPLC–MS. Owing to the single-step liquid-liquid extraction and mass detection, it is simple, rapid, and highly specific and sensitive. Up to now the cost of the equipment constituted a limiting factor, however an increasing number of reports over the past five years seem to indicate that MS (especially with API interfaces) may represent the likely future of HPLC

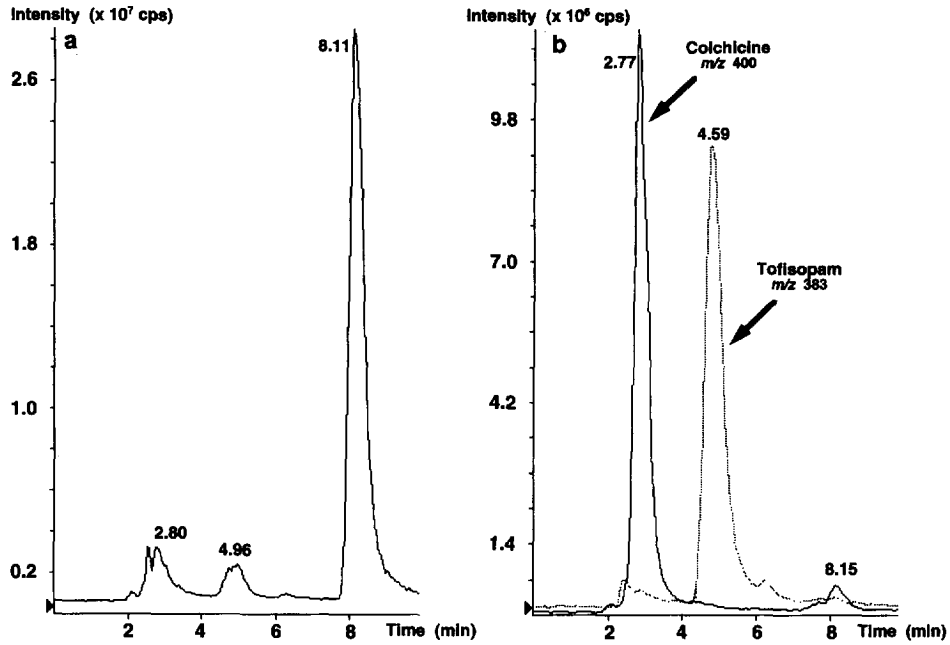

Fig. 5. (a) HPLC–ISP-MS chromatogram from a blood extract (1-ml sample) in a suspected fatality; TIC recording (m/z 380–405); Chromatographic conditions as in Fig. 4. (b) HPLC–ISP-MS reprocessed chromatogram, extracted from data shown in Fig. 5a by selecting m/z 383 and 400. Measured COL concentration: 96 ng/ml blood.

detection – or at least the alternative of choice to UV in all situations requiring both unequivocal identification and high sensitivity for compounds not amenable to GC. This paper presented a preliminary application of such a technique. Further results will be subsequently published.

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