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Quantitative high-performance liquid chromatographic determination of acrolein in plasma after derivatization with Luminarin® 3

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Abstract

A rapid, sensitive and specific high-performance liquid chromatographic method for the quantification of acrolein (**1**), one of the toxic metabolites of oxazaphosphorine alkylating agents (cyclophosphamide and ifosfamide) was developed. Condensation of acrolein with Luminarin® 3 afforded a fluorescent derivative that could be specifically detected and quantified. Chromatographic conditions involved a C_{18} RP column Uptisphere and a gradient elution system to optimize resolution and time analysis. The method showed high sensitivity with a limit of detection of 100 pmol/ml and a limit of quantification of 300 pmol/ml. This technique is particularly suitable for pharmacokinetic studies on plasma of oxazaphosphorine-receiving patients.

Keywords: Acrolein; Ifosfamide; Cyclophosphamide; Luminarin

1. Introduction

Oxazaphosphorine drugs are alkylating antineoplastic substances used in various cancer chemotherapy regimens. Cyclophosphamide (CPM, **2**) and ifosfamide (IFM, **3**) are members of this family and are widely used for the treatment of sarcoma [1,2]. More particularly, high doses of IFM (up to 9 g/m^2) are sometimes administered to children suffering from osteosarcoma and soft tissue sarcoma [3,4]. Actually, IFM and CPM are non-cytotoxic prodrugs that require a bioactivation step that occurs in the liver via a cytochrome P450-mediated ring oxidation. Indeed, after oxidation at position 4, true alkylating moieties (phosphoramide (**4**) or isophosphoramide (**5**) mustards) are spontaneously and concomitantly formed with the release of acrolein (Fig. 1). This latter is responsible for the urotoxic side effects of the therapy [5,6] and its involvement in CPM-induced cellular toxicity has also recently been proposed by Friedman et al. [7]. Despite the use of large quantities of mesna (sodium mercaptoethanesulfonate) to prevent acrolein toxicity, haemorrhagic cystitis [1–4] frequently occurs with alarming severity. Since oxazaphosphorine metabolism is sensitive to auto-induction [8], as well as to inter- and intra-

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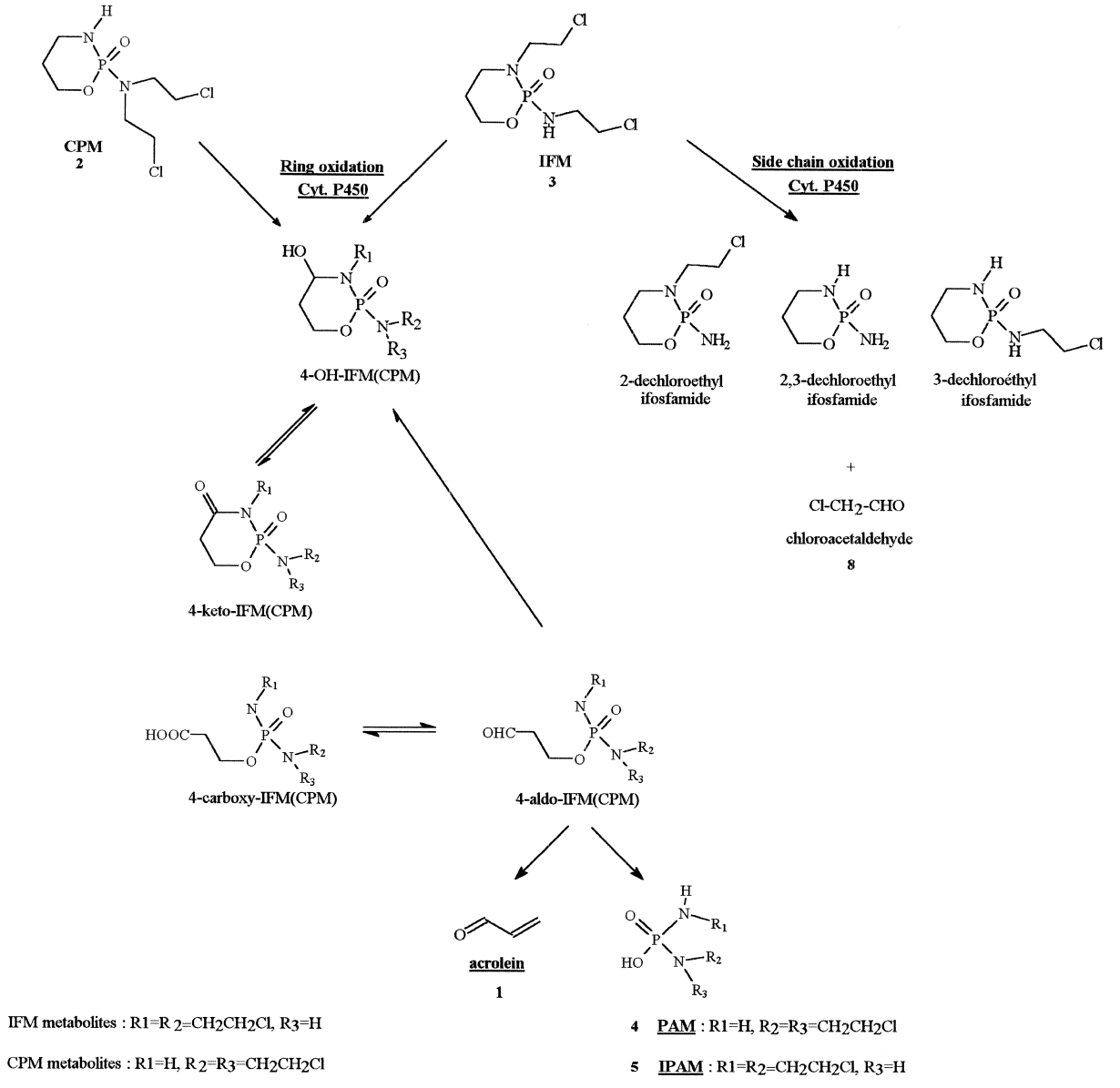

Fig. 1. CPM and IFM metabolism pathways.

individual variations [9], an accurate measurement of acrolein levels in plasma should facilitate individual pharmacokinetic and metabolism studies of these drugs and thus lead to a better understanding of the variation in the metabolism. For these reasons, the monitoring of the acrolein level in plasma is necessary to efficiently adjust the mesna dosage and consequently to optimize IFM and CPM use.

Different methods have been described to achieve the quantitative determination of acrolein in ambient air [10,11] or in biological samples or urine [12–14]. Among these methods, the formation of an hydroxyquinoline by condensation of acrolein with 3-aminophenol allowing fluorimetric detection after HPLC has been reported [12,13]. However, this method, only described for urine or liver microsome extracts

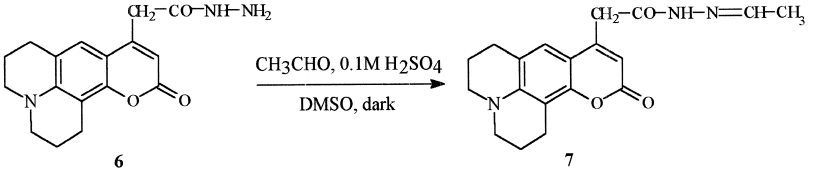

Fig. 2. Synthesis of Lum-acet.

analysis, requires drastic conditions (heating of the samples at 100°C) that might modify the real kinetics of the aldehyde formation, by a thermodynamically induced mecanism, in the plasma of patients receiving multitherapy. Another HPLC method including the derivatization of acrolein with 2,4-dinitrophenylhydrazine [14] leading to UV detection has also been proposed but the necessary 254 nm detection dramatically limits its sensitivity and specificity in the case of plasma samples. Facing the crucial need of possessing an accurate method of the quantification of acrolein in plasma, we were eager to develop an HPLC technique with fluorescent detection using only mild conditions and hence suitable for the quantitative determination of acrolein in human plasma.

A few years ago, we demonstrated the favourable fluorescent properties of Luminarin® 3 (1H,5H,11H-[1]benzopyrano[6,7,8-*ij*]quinolizine-9-acetic acid 2,3,6,7-tetrahydro-11-oxohydrazide) (**6**) (Fig. 2) derivatives of carbonyl compounds allowing the detection of acrolein at the pico scale [15,16]. This method being carried out at room temperature, we consequently tried to apply this methodology and have been able to adapt it for the detection of acrolein in plasma samples. In order to prepare a suitable I.S., we studied several other aldehydes and chose the condensation adduct of Luminarin® 3 and acetaldehyde as an I.S. (**7**) (Fig. 2).

2. Experimental

2.1. Apparatus

Mass spectrometry (MS) was performed with a Nermag R-1010 instrument. NMR spectra were performed on a Bruker AC 300-P spectrometer.

LC analyses were performed with a Beckman (Gagny, France) 126 binary pump equipped with a Beckman 210A injector, a 20-μl sample loop and a Jasco 820-FP fluorescence LC detector. λ_{exc} and λ_{em} were set at 407 and 507 nm, respectively (attenuation was 64 and Gain ×1). The LC system was driven by software GOLD 8.1 Beckman.

RP chromatography was performed on a 150×4.6 mm I.D., 5 μm ODS Uptisphere column (Interchim, Montluçon, France) using acetonitrile–0.01 *M* imidazole buffer, pH 7.5 (30:70, v/v) as initial mobile phase and an elution gradient was used during the analysis. The flow-rate was set at 1.8 ml/min and kept constant during the analysis.

2.2. Reagents

All reagents and solvents (LC and fluorimetric grade) were purchased from Aldrich (St. Quentin Fallavier, France) or Sigma (St. Louis, MO, USA) except Luminarin® 3 which was supplied by Seratec (Epinay sur Seine, France). Stock solution of Luminarin® 3 in dimethyl sulphoxide (DMSO) were diluted further with the same solvent to the required concentrations. All solvents used were carefully checked for the absence of fluorescent impurities.

2.3. Acid acrolein (pro-2-enal) and acetaldehyde solutions

Solutions (0.1 m*M*) of each aldehydes, in 1% sulfuric acid, were prepared and diluted when necessary with the same solvent to the desired concentration. Stock solutions in DMSO were kept frozen at −20°C.

2.4. Synthesis of the luminarin® 3 derivatives

Luminarin® 3 derivative of acrolein (Lum-acro) was obtained according to Traoré et al. [15]. For the preparation of a Luminarin® 3 derivative of acetaldehyde, a solution of Luminarin® 3 (313 mg, 1

mmol) in 10 ml of DMSO was added to a solution of aldehyde (1 mmol in 100 ml of 1% sulfuric acid). The reaction was carried out in the dark, for 1 h at room temperature.

Sodium hydrogen carbonate (2.5g) was added to adjust the pH of the reaction mixture to ~7.3. The Luminarin® 3 derivative was extracted twice with 50 ml of methylene chloride. The organic phase was dried by the addition of 10 g of anhydrous $MgSO_4$, then evaporated to dryness under vacuum. The residue, dissolved in 5 ml of methylene chloride, was purified by means of column chromatography (25× 3.5 cm I.D. column) on silica gel 60 (0.04–0.063 mm, Merck, Darmstadt) with a methylene chloride–methanol gradient as eluent to give Luminarin® 3-acetaldehyde (Lum-acet) (204 mg, 60%).

Luminarin® 3-acetaldehyde (yellow powder); CI-MS, m/z 357 [$M+NH_4^+$], 340 [MH^+], 256. ^{1}H-NMR [$CDCl_3$–CD_3OD (70:30, v/v)] δ_H (ppm); 1.78 (m, 7H); 2.65 (m, 4H); 3.10 (m, 4H); 3.43 (s, 1H); 3.84 (s, 2H); 5.82 (s, 1H); 6.93 and 6.95 (s, 1H, *syn* and *anti* forms), 7.15 and 7.33 (q, J=5.4 Hz, 1H, *syn* and *anti* form). ^{13}C-NMR [$CDCl_3$–CD_3OD (70:30, v/v)], δ_C: 17.6 (CH_3), 19.9 (CH_2), 20.2 (CH_2), 21.1 (CH_2), 27.3 (CH_2), 35.4 (CH_2), 49.1 (CH_2), 49.5 (CH_2), 106.3 (C), 107.8 (CH), 108.1 (C, CH), 118.2 (C), 121.7 (CH), 144.7 (2 C), 150.8 (C), 162.8 (C), 170.4 (C).

UV and fluorimetric spectra gave, respectively, $\lambda_{exc(max)}$ (nm) (water–acetonitrile) 410/391 and λ_{exc}/λ_{em} (nm) (water) 392/508 and (acetonitrile) 392/467. The UV and fluorescence spectral data of each compound was established in water (pH 1.5, 2.5, 4, 6, 8, 10) and in acetonitrile (Table 1). Optimal pH conditions were found to be between 6 and 8. Optimal wavelength conditions were found to be 407 and 507 nm for λ_{exc} and λ_{em}, respectively.

Table 1
UV and fluorescence spectral data of Luminarin 3®, Lum-acro and Lum-acet

	Solvent	Absorbance $\lambda_{exc(max)}$ (nm)	Fluorescence $\lambda_{exc}/\lambda_{em}$ (nm)
Luminarin® 3	Water	408	403/502
	Acetonitrile	391	394/464
Lum-acro	Water	408	392/507
	Acetonitrile	391	392/468
Lum-acet	Water	410	392/508
	Acetonitrile	391	392/467

2.5. Derivatization procedure

Derivatization of acrolein in the plasma was carried out by adapting our previously reported procedure [15,16]. All samples were prepared in a drug-free human plasma spiked with a 0.1 m*M* working solution of aldehyde in 0.1 *M* sulfuric acid.

A 1-ml plasma spiked with 0.5–20 nmol of acrolein in 0.1 *M* sulfuric acid was mixed with 100 μl of the latter and 10 μl of a 5 m*M* Luminarin® 3 solution. The mixture was kept at room temperature for 1 h in the dark. As an I.S., 20 μl of a 0.5 m*M* Lum-acet solution in DMSO was added. A blank sample tube of plasma was prepared with Luminarin® 3 and I.S. only. A 100-μl volume of 0.6 *M* aqueous sodium hydrogen carbonate solution was carefully added to adjust the pH to ~7.0 and the tube was agitated until emitting of gas ceased. Methylene chloride (2 ml) was then added and the mixture was horizontally and vigorously agitated for 2 min. The layers were separated by centrifugation (3 min, 5000 rpm) and the organic layer was collected (1.8 ml), transferred to a clean tube and evaporated under a gentle stream of nitrogen at room temperature. The resulting residue was dissolved in 100 μl of acetonitrile and an aliquot (20 μl) was injected into the chromatograph.

3. Results

3.1. Structure of lum-acet

The structure of Lum-acro has already been established in our previous work [13]. The structure of Lum-acet was confirmed by MS, ^{1}H-NMR and ^{13}C-NMR. Its mass spectrum (CI) displayed a quasi-molecular peak at m/z 357 [$M+NH_4^+$] and a molecular peak at m/z 340 [MH^+]. Unambiguous evidence for the formation and purity of the lum-acet was confirmed by LC, and NMR spectral data (see Experimental). In the ^{1}H-NMR spectrum, the *syn*/*anti* forms of Lum-acet were responsible for a 1:1 splitting of H8 and H5′. However, the LC analysis of

Lum-acet showed a single peak (t_R 10.0 min), the excess of Luminarin® 3 giving a peak at 4.4 min.

3.2. Elution gradient and LC separation

Although both normal and RP HPLC have been shown to be suitable for the chromatography of lum 3-aldehydes [15,16], for our study, we chose to test only RP columns, in order to rapidly elute the hydrophilic products contained in plasma. The separations achieved between Lum-aldehydes and Luminarin® 3 using Spherisorb, LiChrospher, and Nucleosil columns were not efficient enough to allow an accurate resolution, but an Uptisphere C_{18} column (RP), whose silica is deactivated, gave good separation between lum 3-acrolein and Luminarin® 3. Fig. 3 shows HPLC profiles of blank plasma, plasma spiked with 10 nmol/ml of acrolein and 10 nmol/ml of I.S. and plasma of a patient who received 3 g/m^2 of ifosfamide over a 5-day infusion period. The elution sequence was Luminarin® 3, Lum-acro and Lum-acet. Retention times were 4.4, and 7.1 min for Luminarin® 3 and Lum-acro, respectively, and the resolution between the two compounds was 2.3.

Under the same chromatographic conditions, the retention time of Lum-acet was 10.0 min (Fig. 3) a delay which made it suitable for use as an internal standard (I.S.). The resolution between Lum-acro and Lum-acet was 2.4.

The elution gradient diagram is shown in Fig. 4. The initial mobile phase consisted of acetonitrile–0.01 *M* aqueous imidazole buffer (30:70, v/v). The proportion of acetonitrile was kept at 30% (v/v) for 1.8 min. Between 1.8 and 2.1 min, the acetonitrile proportion was decreased to 23% (v/v) and kept until $t=4.5$ min in order to optimize the separation of the Luminarin® 3 and the Lum-acro. At $t=4.5$, the acetonitrile proportion was increased to 35% (v/v) in 0.75 min ($t=5.25$ min). This proportion was maintained for 6.75 min ($t=12$ min). Then, the organic phase was increased up to 40% (v/v) over 1 min and kept constant for 5 min ($t=18$ min). This elution gradient permitted a shortening of the time analysis owing to more rapid elution of an additional compound ($t_R=18$ min). This latter is probably the compound resulting from the condensation of Luminarin® 3 and the dimer of acrolein as the peak increased with the increasing amount of acrolein [15]. A 2-min gap was scheduled to restore the initial conditions.

3.3. Validation

3.3.1. Specificity

The specificity of an analytical method corresponds to its ability to accurately measure an analyte in the presence of interferences that may be present in the sample matrix. The R_s values calculated for each compound against a second one were greater than 2, highlighting the good specificity of this HPLC method.

3.3.2. Linearity

The linearity of the assay was established from 0.5 to 20 nmol (28–1120 ng/ml) of acrolein per millilitre of plasma by preparing standard curves on 6 different days. The calibration graph was linear in this range with a mean correlation coefficient >0.985. A typical equation was $y=0.1171x+0.0691$ with $n=6$, where y is the peak height ratio of Lum-acro over the I.S., and x the acrolein concentration.

3.3.3. Recovery

The extraction recoveries of Lum-acro and Lum-acet from human plasma were calculated from comparison with the peak height of directly injected Lum-acro (Lum-acet) standards and those from plasma spiked with the same compounds submitted to the extraction procedure ($n=6$). The extraction recoveries of Lum-acro (10 nmol/ml) and Lum-acet (10 nmol/ml) were 78% and 82%, respectively.

3.3.4. Precision

The precision of a quantitative method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings. It is measured by successively analyzing samples and expressed as relative standard deviation (RSD%) of the results. The intra-assay (within-day) and inter-assay (between-day) precision of the described assay were examined by supplementing pooled blank plasma with two amounts of acrolein (i.e. 1 and 20 nmol/ml), replicated six times. Each sample was prepared independently and analyzed

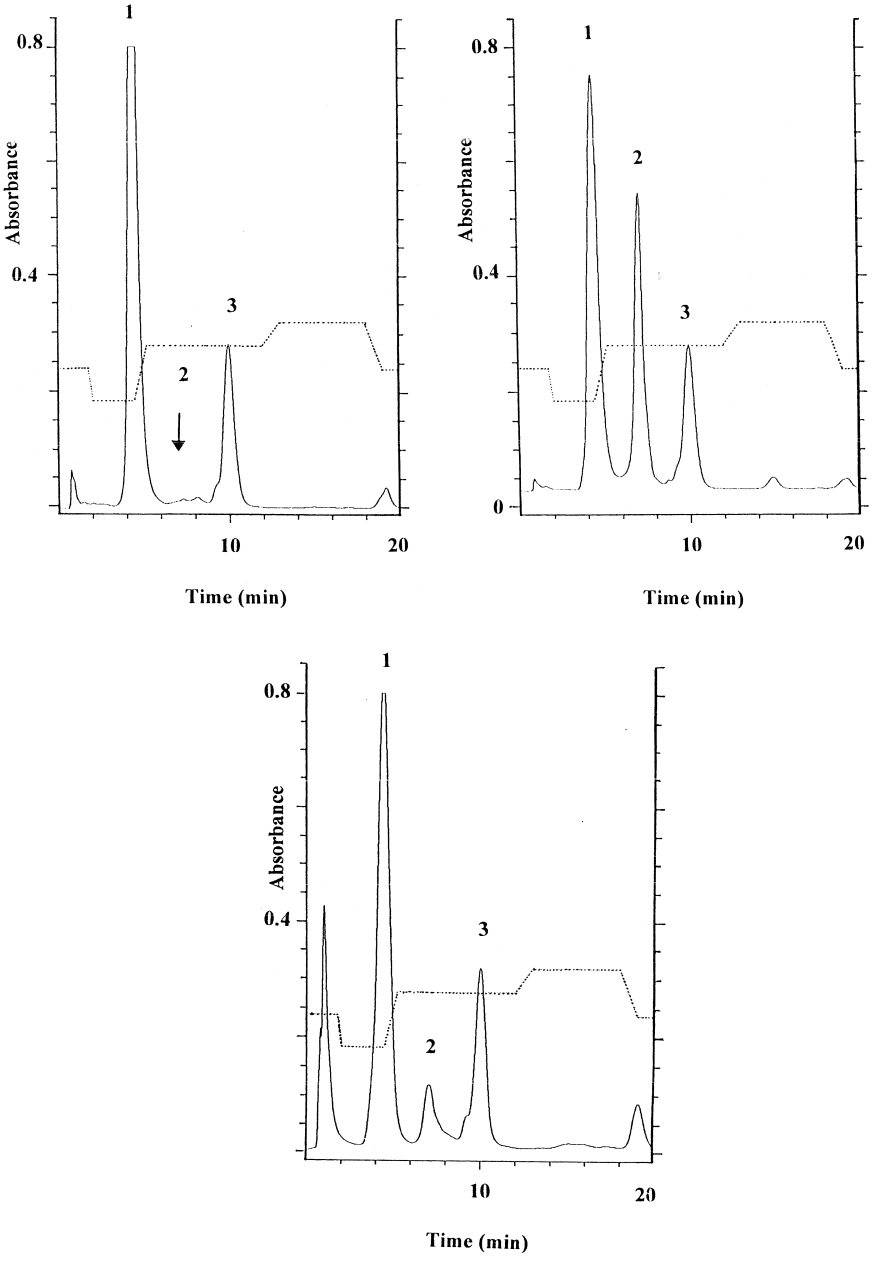

Fig. 3. RP HPLC chromatograms of plasma samples. (a) Blank plasma spiked with Luminarin® 3 (10 nmol/ml) and I.S. (**3**) (10 nmol/ml); (b) Plasma sample containing 10 nmol/ml of acrolein (Lum-acro **2**) spiked with excess of Luminarin® 3 and I.S. (10 nmol/ml); (c) Plasma sample from a patient after i.v. administration of 3 g/m^2 of IFM. Dashed line (. . . . .) corresponds to the elution gradient.

according to the described procedure. The RSD values for the intra- and inter-assays are presented in Table 2.

3.3.5. Limit of derivatization

When developing a derivatization procedure, it is of major importance to assess the lowest quantity of

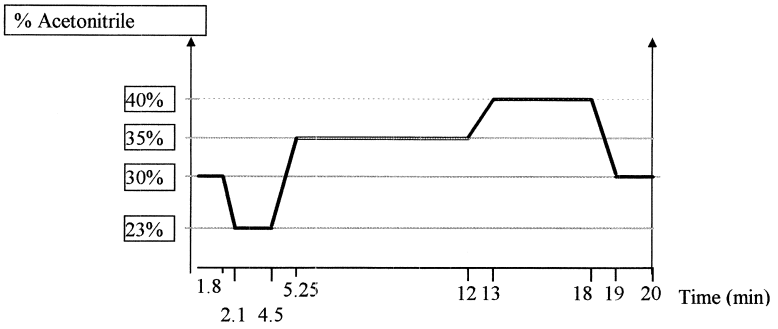

Fig. 4. Elution gradient system used in this analytical technique.

compound that can be derivatized which could influence the limit of detection. Under our chromatographic conditions, we could clearly identify a peak at the retention time of Lum-acro significantly different from the blank plasma spiked with an amount of acrolein as low as 100 pmol/ml.

3.3.6. Limits of detection and quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of acrolein were obtained by use of the slope (b) and the standard deviation of the intercept (SDa) from six calibration graphs determined by linear regression line as defined by IUPAC [17] and ICH Topic Q2B [18]. The LOD calculated ($LOD = 3.3 \times SDa/b$) was 100 pmol/ml (5.6 ng/ml). The LOQ ($LOQ = 10 \times SDa/b$) was 300 pmol/ml (16.8 ng/ml).

3.4. Discussion

The procedure described is a fluorimetric–HPLC method for the quantitative determination of acrolein in plasma with Lum-acet as the I.S. To choose the I.S., in addition to Lum-acet, we synthesized, according to previously reported procedures [15] five derivatives obtained by condensing Luminarin® 3 and malonaldehyde, methylmalonaldehyde, 2-methylbutyraldehyde, isobutyraldehyde and furfuraldehyde. Then, we evaluated the chromatographic and fluorimetric properties of each compound. Owing to their lipophilic properties, all the aldehydes derivatizated had longer retention times than Lum-acro. Malonaldehyde, methylmalonaldehyde, furfuraldehyde are the aldehydes that lead, after condensation with Luminarin® 3, to schiff bases that further yield to hydroxy-pyrazole and then pyrazole. Since this reaction is difficult to complete we did not further consider their use as I.S. Isobutyraldehyde and 2-methylbutyraldehyde each afforded only one derivative but of too long retention times (>15 min) for a routine analysis.

Lum-acet exhibited adequate chromatographic properties with a retention time of 10 min. Furthermore, it is easily synthesized and leads to an amorphous yellow powder that is handy to use in practice.

Since we were designing a method for the quantification of acrolein in plasma of patients receiving IFM and CPM, the second point we had to deal with was the possible presence of two aldehyde derivatives in the samples: acrolein and chloracetaldehyde (**8**) (Fig. 1). Chloracetaldehyde is a metabolite specifically found in the plasma of patients receiving a high dose of IFM but is rarely metabolized from CPM. To demonstrate the lack of interference of chloracetaldehyde in the quantification of acrolein, we specifically prepared the schiff base resulting from the condensation of chloracetaldehyde and Luminarin® 3 under the conditions used to prepare

Table 2
Inter- and intra-assay precision data

Amount (nmol)	Intra-assay (%)	Inter-assay (%)
1	7.0	8.6
20	5.4	8.1

Lum-acro. Hence, we were able to characterize Luminarin® 3 derivative of chloracetaldehyde (Lum-Cl) (data not reported) and demonstrated that the schiff base is the only adduct obtained (no product resulting from a nucleophilic substitution of the Luminarin® 3 on the chloromethylene group was observed or no dimer compound). Unfortunately, and in contrast to Lum-acro, a strong quenching of the fluorescence was observed for Lum-Cl, likely due to the presence of the chlorine atom [19]. Under our conditions, preliminary chromatographic assays allowed us to identify the peak corresponding to Lum-Cl retention time (9 min) and show that it did not interfere with either Lum-acro or Lum-acet.

4. Conclusion

Fluorimetric detection is highly sensitive and its use has already permitted the design of numerous methods to specifically quantify metabolites in mixtures. In this paper, we have demonstrated that our previous reported method for the quantification of aldehydes can easily be adapted to allow quantitative determination of acrolein in plasma.

Our method does not require heating of the sample and consequently the risks of modified kinetics, as well as those of denaturation or artefact production are virtually eliminated. Under the conditions reported acrolein as a IFM or CPM metabolite can be specifically quantified in plasma. Application of our method to the quantification of chloracetaldehyde is being carried out.

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