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Quantification of fumaric acid in liver, spleen and urine by high-performance liquid chromatography coupled to photodiode-array detection

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ABSTRACT

Quantification of fumaric acid, an endogenous dicarboxylic acid with interesting biomedical applications either through its own biological activity or as a linker constitutive of the porous iron(III) fumarate metal organic framework (MOF) MIL-88A based drug nanocarrier (MIL stands for Material from Institut Lavoisier), has been developed in different rat biological complex media (liver, spleen and urine). After a liquid–liquid extraction procedure, fumaric acid concentration was determined by a simple and accurate high-performance liquid chromatography (HPLC) method coupled to a photodiode-array detector (PDA) using aminosalicylic acid as internal standard (IS) and a gradient elution. The recovery of fumaric acid reaches 89% and 92% for urine (for concentrations of 0.05 and 1 $\mu g\,ml^{-1}$, respectively) and 90% for liver and spleen tissues, exceeding 89% in all instances in comparison with the IS. Linearity has been kept from 0.05 to 1 $\mu g\,ml^{-1}$ and from 0.5 to 10 $\mu g\,g^{-1}$ of fumaric acid in urine and tissues, respectively. The limit of detection of the method was 0.01 μg per injection. This method has finally allowed the quantification of fumaric acid in rat urine and tissue samples after the intravenous administration of MIL-88A nanoparticles.

1. Introduction

Fumaric acid (Fum) is both a key intermediate in the citric acid cycle (Krebs cycle) for energy production within the cell and a product of the urea cycle in mammalians [1]. This endogenous unsaturated dicarboxylic acid possesses interesting biological effects such as antipsoriasis, anti-inflammatory, neuroprotective and chemopreventive activity. A therapy containing fumaric acid esters (FAEs; dimethyl fumarate and ethylhydrogen fumarate, called Fumaderm®) is safe and effective in patients with severe psoriasis vulgaris [2]. Although the mechanism is not well-known, FAEs are involved in the expression of anti or pro-inflammatory cytokines (IL-10, IL-4, IL-5 or IL-6, IL-1β, TNF-α, respectively) and in the alteration of leukocyte, keratinocyte and/or endothelial functions [3]. FAEs also exert neuroprotective effects (neuroinflammation, autoimmune encephalomyelitis) [4].

Attractive chemopreventive potential effects of fumaric acid on lung adenomas [5] as well as of fumarate derivatives on hepatocarcinomes [6] have also been reported. Fum does also reduce the hepatocarcinogenicity of mitomycin C and aflatoxin B1 [7] as well as of aflatoxin B1, 3′-methyl-4-(dimethylamino)azobenzene and thioacetamide [7]. Besides, Fum is used as food additive (E297) in many processed foods to keep them stable and control their acidity [8]. Moreover, the hydrophobic nature of fumaric acid makes it an effective antimicrobial agent for fruits and vegetables preservation [9].

Fum is currently studied as an experimental drug (EXPT01460, EXPT01511) [10] due to its action on different targets such as malate dehydrogenase, fumarate reductase flavoprotein subunit, mitochondrial NAD-dependent malic enzyme and fumarylacetoacetase [11].

On the other hand, we have recently reported novel drug nanocarriers with interesting imaging properties based on porous iron(III) MOFs [12–14]. This type of versatile highly porous crystalline hybrid solids [15], offers many possibilities in biomedicine, such as drug carriers, release of biological gases or imaging [16,17]. Specifically, the porous iron fumarate MIL-88A, built up from oxocentered trimers of iron (III) octahedra connected by fumarate anions that delimit a three dimensional pore system ($\phi \sim 5–7$ Å), possess many interesting features. First, it exhibits a highly flexible framework with changes in the unit cell volume above 80% upon adsorption–desorption of polar solvents as well as the presence of a large amount of metal Lewis acid sites within its three-dimensional framework [18]. Secondly, nanoparticles of MIL-88A (MIL-88A_nano) [19] have also shown an excellent

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biocompatibility associated to significant drug loading capacities and interesting imaging properties [14,20].

The above mentioned biological applications, including therapeutic activity of Fum by itself or by its use as a constituent of a drug nanocarrier, make the determination of fumaric acid concentration in biological matrices an important issue. In this regard, different methodologies have been applied to the Fum determination. For instance, the concentration of Fum produced by *Rhizopus* and *Aspergillus* microorganisms, has been quantified by different chromatographic methods (Ion Chromatography, Ion-Exclusion or Reverse-Phase) and detection techniques (UV–vis absorption or conductimetry) [21–23]. Likewise, Fum concentration has been determined using gas chromatography coupled with mass spectrometry either in drinks [24] or tissues [25] and by gas chromatography with capillary column coupled with flame ionization detection in human serum and urine [26]. Subsequently, a HPLC method has been successfully applied for the determination of Fum in rat biological matrices (plasma, urine and fecal samples) [27]. However, the use of isocratic elution, described herein, limits the method efficiency to analyze Fum in more complex biological tissues such as liver or spleen, which are known to be the nanoparticles target organs [14]. Therefore, here we describe a convenient liquid–liquid extraction procedure followed by a simple, sensitive and reproducible HPLC method coupled with a UV–vis spectroscopic detection (PDA) to determine Fum concentration in different biological complex media (i.e. liver, spleen and urine).

2. Experimental

2.1. Reagents

All the reactants, including HPLC grade methanol, fumaric acid, aminosalicylic acid, sodium dodecyl sulfate (SDS), sodium hydrogen phosphate heptahydrate ($Na_2HPO_4{\cdot}7H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$) and orthophosphoric acid, have been purchased from Sigma–Aldrich (France). The phosphate buffer 0.04 M is prepared in ultra pure water by mixing $Na_2HPO_4{\cdot}7H_2O$ and $NaH_2PO_4{\cdot}2H_2O$. The pH is adjusted to 2.5 or 5 by adding some drops of orthophosphoric acid. Standard solutions of Fum (denoted S; $S = 100\ \mu g\ ml^{-1}$) and aminosalicylic acid, used as internal standard (denoted IS; $IS_1 = 1\ \mu g\ ml^{-1}$ and $IS_2 = 10\ \mu g\ ml^{-1}$), were prepared in methanol and stored at 4 °C.

2.2. Liquid chromatography conditions

UV–vis spectra of Fum and the IS have been collected using standard solutions S and IS_1 in a Shimadzu UV-160 A spectrophotometer. Maximum absorption wavelengths for Fum and IS are 215 and 320 nm, respectively. Fum concentration was determined using a RP-HPLC system (Reversed phase-HPLC) Waters Alliance C2695 separation module (Waters, Milford, MA, USA) equipped with a photodiode array detector (PDA) Waters E2998 and controlled by Empower software. The analytical column was a RP-C_{18} 5 μm Sunfire 150 mm × 4.6 mm I.D. (Waters, France) protected by HPLC cartridge precolumn C_{18} 5 μm Sunfire 20 mm × 4.6 mm I.D. (Waters, France). Composition of the mobile phase, a solution of methanol in phosphate buffer 0.04 M pH = 2.5 (phosphate pH 2.5), has been changed depending on the biological media (liver, spleen and urine). For liver and spleen samples, the separation of Fum and IS was optimized using a linear gradient elution from 5:95 to 50:50 (v/v) methanol–phosphate pH 2.5 (0–5 min) followed by an isocratic mode 50:50 (v/v) methanol–phosphate pH 2.5 (5.1–10 min). The flow rate was 0.8 ml min^{-1} and the injection volume 50 μl.

For urine samples, the applied conditions consisted in a linear gradient from 5:95 to 50:50 (v/v) methanol–phosphate pH 2.5 (0–4 min) followed by an isocratic step 50:50 (v/v) methanol–phosphate pH 2.5 (4.1–9 min). The flow rate was 0.8 ml min^{-1} and the injection volume of 50 μl. The column was finally washed and reconditioned during 20 min. In all cases the column has been operated at 37 °C and both, Fum and IS were monitored and quantified at 215 nm.

2.3. Animal preparation

All experimental procedures have been reviewed and approved by the Animal Experimentation Ethics Committee of the Faculty of Pharmacy of Paris-Sud 11 University, France. The study has been performed using female Wistar rats (body weight 110 ± 10 g) obtained from the central animal care facilities, Janvier R Center d'Elevage, France.

In order to determine the Fum in liver, spleen and its excretion from urine, 220 mg kg^{-1} of nanoparticles of MIL-88A [19], were suspended in a 0.5 ml aqueous solution of glucose (10%) and intravenously perfused [24] in the jugular vein under isofluorane anesthesia to a group of 6 rats (denoted MIL-88A group) [14]. Likewise, a control group (named Glu group) was perfused in the jugular vein with 0.5 ml of a glucose solution 10%. 24 h after treatment, all animals were sacrificed under isofluorane anesthesia. Spleens and livers samples were extracted, washed with a NaCl 0.9% solution and stored at −20 °C until HPLC analysis. Urine samples, collected during the whole time course of the experiment, were centrifuged (8000 g/15 min) and stored with 0.1 ml of H_2SO_4 0.01 M per milliliter of urine at −20 °C until HPLC analysis.

2.4. Determination of fumaric acid in urine

A urine sample (0.5 ml) was centrifuged at 8000 × *g* for 10 min and the supernatant filtered through a 0.2 μm sterile syringe filter into an Eppendorf tube and half diluted in phosphate buffer at pH = 5. In a 5 ml glass tube containing 0.5 ml of the diluted urine sample, 0.1 ml of IS_1 was added. After shaking for 1 min, 2 ml of a mixture of methanol and phosphate buffer, v/v (0.04 M, pH = 2.5) was added, and stirred in darkness at room temperature for 30 min. After centrifugation at 8000 × *g* for 10 min, 0.1 ml of the supernatant was diluted by half in mobile phase, right before the injection in the HPLC system.

2.5. Determination of fumaric acid in tissues

1 g of liver taken from the right lobe or 0.1 g of spleen taken from right extremity were added to 0.5 ml of SDS 0.1 M and 0.1 ml of IS_2 in 10 ml glass tubes. After homogenization using a dispersing ultra-turrax, 1 ml of phosphate buffer (0.04 M, pH = 2.5) was added and the extraction was performed 2-times by adding 1.5 ml of methanol and 1 ml of acetonitrile after 5 min later sonication. Then, the mixture was shacked and stirred for 1 h in darkness at room temperature. After centrifugation at 8000 × *g* for 10 min, the supernatant was collected and filtered through a 0.2 μm sterile syringe filter. In order to avoid an eventual partial extraction of the fumaric acid, the pellet was treated again under the same conditions. Then, the supernatant of each extraction was evaporated under a nitrogen flow and the dry residue dissolved in 0.5 ml of methanol. Finally, the extract was diluted in the mobile phase before the injection into the HPLC system.

2.6. Validation of the method and quality control samples

The stock solution of Fum ($S = 100\ \mu g\ ml^{-1}$) has been diluted to give a series of standard solutions with concentrations ranging from 0.05 to 10 $\mu g\ ml^{-1}$. For livers and spleens, linearity, accuracy and method recovery were studied by spiking homogenized biological

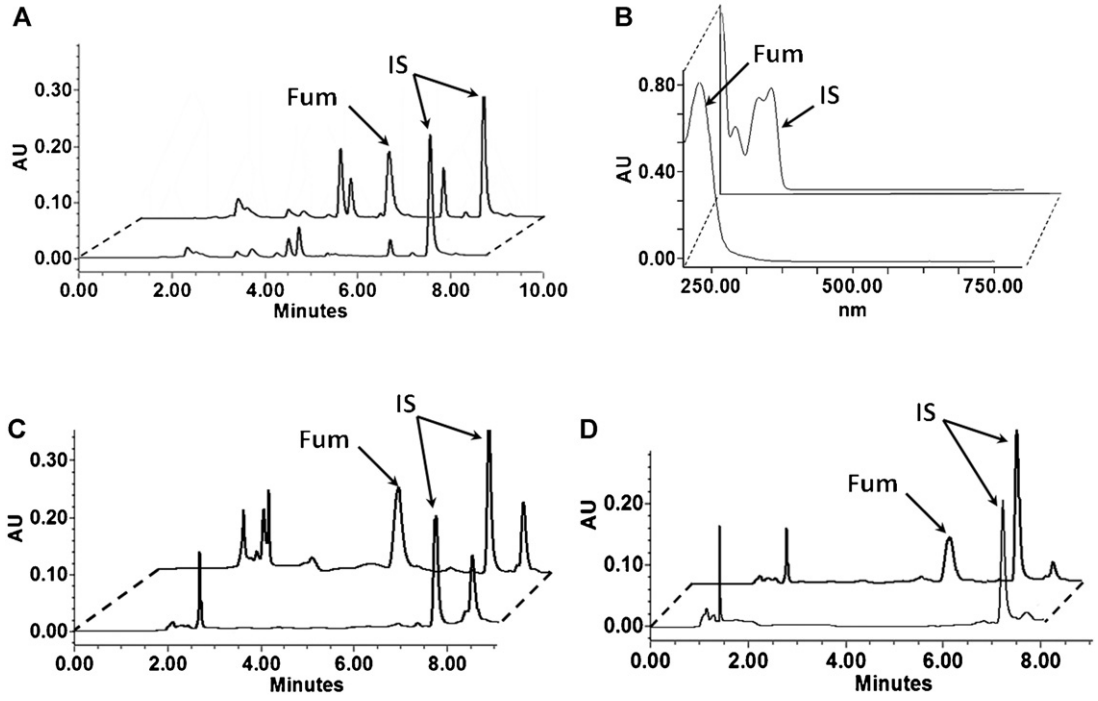

Fig. 1. Chromatograms of extracts of control (in front) and treated with MIL-88A_nano (back) liver (A), spleen (C) and urine (D) samples. UV–vis spectra of internal standard (IS) and fumaric acid (Fum) obtained by PDA detection at 215 nm (B).

samples (1 g of liver or 0.1 g of spleen + 0.5 ml of 0.1 M SDS) collected from control animals, with 0.1 ml of different concentrations of Fum (ranging from 0.5 to 10 $\mu g\,ml^{-1}$). In the same way, urine samples of control animals (0.5 ml of urine were centrifuged at 8000 × *g* for 10 min; the supernatant was filtered through a 0.2 μm sterile syringe filter into an eppendorf tube and diluted in phosphate buffer at pH = 5) were spiked with 0.1 ml of different concentrations of Fum ranging from 0.05 to 1 $\mu g\,ml^{-1}$. In order to check the specificity of the method, the peak purity of Fum was investigated using the photodiode array detector and the retention time.

Quality control samples (QCs) were prepared by spiking liver, spleen and urine from untreated rats with different Fum concentrations (0.05, 0.2 and 1 $mg\,ml^{-1}$ for urine samples and 0.5, 5 and 10 $mg\,g^{-1}$ for liver and spleen tissues).

3. Results

The extraction recovery of Fum from tissues and urine was determined by spiking samples collected from untreated animals with Fum at low concentrations (0.05 and 1 $\mu g\,ml^{-1}$ for urine; 0.5 and 10 $\mu g\,g^{-1}$ for liver and spleen tissues, number of samples $n = 6$). Recoveries were calculated by comparing the peak areas with those obtained after direct injection into the chromatograph using the corresponding pure solutions. The recovery of fumaric acid reached 89% and 92% for urine (for concentrations of 0.05 and 1 $\mu g\,ml^{-1}$, respectively) and 90% for tissues. The coefficient of variation (CV) did not exceed 5% in all cases. The recoveries calculated regarding the IS for both upper and lower values exceeded 95% in both cases.

The chromatograms of biological samples of control and treated rats obtained by the HPLC–PDA method are shown in Fig. 1A, C and D. The identity of Fum was ascertained by its characteristic UV–vis spectra with a maximum absorption wavelength at 210 nm (Fig. 1B). Chromatograms profiles of liver (A), spleen (C) and urine (D) extracts, obtained from the control and the MIL-88A_nano treated rats, did not exhibit any peak interfering with IS (retention time of IS and Fum peaks at 7.40 and 5.85 min, respectively). The limit of the detection of the method (signal/noise ratio = 3) corresponded to 0.01 μg of injected fumaric acid. Both sensitivity and specificity of PDA mode were sufficient to determine the concentration of Fum in tissues and urine.

The linearity of the method was checked from 0.05 to 1 $\mu g\,ml^{-1}$. The regression equation for the average calibration curve was $y = 0.0421x + 0.0076$ ($n = 3$, $r = 0.999$), where y is the area Fum/IS ratio; x is the concentration of Fum extract; n is the number of samples and r is the correlation coefficient. For liver and spleen samples, the linearity of the PDA method was confirmed from 0.5 to 10 μg of Fum per gram of tissue, ($y = 0.082x + 0.0136$, $r = 0.997$, $n = 6$ for livers, and $y = 0.076x + 0.029$, $r = 0.994$, $n = 6$ for spleens). For urine, the linearity was checked from 0.05 to 5 μg of Fum per milliliter of urine, ($y = 0.502x + 0.037$, $r = 0.999$, $n = 6$).

Intra and inter-day variability was assessed through the analysis of QCs on the same day and on three different days (Table 1). Accuracy was estimated by comparing the added and the determined concentration of QCs. Precision was determined by calculating the relative standard deviation (RSD) for each concentration of QCs. As shown in Table 1, the intra and inter-day accuracy ranged from 98 to 101% and 96 to 102%, respectively.

Finally, preliminary results of the Fum biodistribution issued from the intravenous administration of MIL-88A nanoparticles to rats are shown in Table 2. Fumaric acid concentrations of the treated rats ranged from 0.945 to 2.002 $mg\,g^{-1}$ (mean = 1.473 $mg\,g^{-1}$), 1.676 to 3.596 $mg\,g^{-1}$ (mean = 2.636 $mg\,g^{-1}$) and from 0.002 to 0.005 $mg\,g^{-1}$ (mean = 0.003 $mg\,ml^{-1}$), for liver, spleen and urine, respectively.

4. Discussion

This study describes a HPLC procedure for the quantification of Fum in biological matrices (rat liver, spleen and urine). Eluent monitoring at 215 nm provides an adequate sensitivity, precision, specificity and accuracy for the identification and determination of Fum in all studied biological matrices. The proposed method based on a gradient elution allows an efficient separation of the internal standard and the Fum. In addition, this method keeps the linearity

Table 1
Precision and accuracy of the HPLC method for fumaric acid determination in rat urine and tissues (spleen and liver).

Spiked samples	Added concentration	Concentration determined within-day ($n = 6$)			Concentration determined between days ($n = 6$)		
		Mean	Accuracy	R.S.D (%)	Mean	Accuracy	R.S.D (%)
Liver 1	0.5 μg g^{-1}	0.506	101.2	5.0	0.510	102.0	5.2
Liver 2	5 μg g^{-1}	4.901	98.0	3.0	4.951	99.0	3.5
Liver 3	10 μg g^{-1}	9.981	99.8	2.0	10.030	100.3	2.2
Spleen 1	0.5 μg g^{-1}	0.502	100.4	4.8	0.488	97.6	2.8
Spleen 2	5 μg g^{-1}	5.010	100.2	2.9	4.971	99.4	4.8
Spleen 3	10 μg g^{-1}	10.030	100.3	1.9	10.070	100.7	2.1
Urine 1	0.05 μg ml^{-1}	0.050	101.0	6.2	0.050	100.0	6.8
Urine 2	0.2 μg ml^{-1}	0.198	99.0	2.5	0.192	96.0	2.4
Urine 3	1 μg ml^{-1}	0.992	99.2	2.6	0.980	98.0	2.8

of Fum within a large range of concentrations (from 0.5 to 10 μg of Fum per gram of tissue and from 0.05 to 5 μg of Fum per milliliter of urine).

Biological sample preparation prior to injection into the chromatograph is an important concern. Several steps, including tissue homogenization, cell lysis, liquid–liquid extraction and dilution, are first required for the correct determination of Fum concentration by HPLC. For an accurate quantification, lysis of cellular membranes with detergents such as SDS has been implemented in order to release the intracellular Fum. In addition, although fumaric acid is quite soluble in water and methanol (6.3 mg ml^{-1} in water at 25 °C) [28], a prolonged contact time under stirring (around 1 h) is however required to fully extract the acid. Fum is then easily separated from the co-extracted lipophilic substances, generally eluted within the solvent front.

For the urine samples, the extraction of fumaric acid is easier due to the higher solubility of its fully deprotonated form in slightly basic urine pH = 8 (pK_a of fumaric acid 3.03 and 4.5) and due to the low complexity degree of the urine matrix when compared to liver and spleen. Indeed, the initial treatment by centrifugation and filtration is efficient to remove most of the impurities present in the urine samples. Thus, only 15 min of stirring are necessary to achieve a significant liquid–liquid extraction of Fum in urine. In comparison with the other reported procedures based on solid phase extractions [25–27], our liquid–liquid extraction method is faster, simpler and easier, providing suitable clean samples with good reproducibility, precision and accuracy. The chromatographic separation optimized by the gradient elution shows a very high selectivity and a good resolution of Fum and IS peaks detected in the different biological complex matrices (Fig. 1A, C and D). Isocratic elution, previously described for plasma, urine and fecal samples [27], is not well-adapted to analyze Fum in complex matrices such as liver or spleen since different extracted compounds might interfere with the Fum or with the internal standard.

Thus, this method has been successfully applied for the identification and quantification of Fum in rats, 24 h after intravenous administration of nanoparticles made of porous iron(III) fumarate MIL-88A (Table 2). Although a significant deviation is observed for the biological samples due to the inter-individual biodegradation, these preliminary results indicate a rapid capture of the MIL-88A nanoparticles by the organs of the Mononuclear Phagocyte System (i.e. liver and spleen). Noteworthy, the here developed HPLC–PDA fumaric acid quantification method, combined with iron determination, pave the way for future biodistribution studies required prior to the use of MIL-88A nanoparticulate systems in drug delivery and/or imaging applications.

Table 2
Fumaric acid concentrations determined in liver, spleen and urine after MIL-88A_nano administration of 220 mg kg^{-1} to rats ($n = 6$).

	Fumaric acid (mg g^{-1})		
	Spleen	Liver	Urine
Mean ± SD	2.636 ± 0.960	1.473 ± 0.529	0.003 ± 0.002

5. Conclusion

A simple and accurate liquid–liquid extraction, separation and quantification method of fumaric acid has been developed. This HPLC/PDA method using gradient elution and aminosalicylic acid as the internal standard, has therefore been applied to the determination of fumaric acid concentration from different biological complex media (liver, spleen and urine). High extraction yield recoveries exceeding 89% are reached, together with a high accuracy of the method. Finally, this method has been successfully applied to analyze the fumaric acid in rat liver, spleen and urine after the intravenous administration of the porous iron fumarate MIL-88A_nano, allowing an in-depth future study of the biodistribution of this MOF nanocarrier.

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