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

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ABSTRACT

The quantification of trimesic acid, a constitutive organic linker from the biodegradable porous iron(III) trimesate MIL-100(Fe) (MIL stands for Materials from Institut Lavoisier), has been performed in different biological complex media (liver, spleen and urine) using a liquid–liquid extraction procedure. A recovery exceeding 92 wt% was achieved from rat tissues and urine spiked with trimesic acid. After extraction, the determination of the trimesic acid concentration was realised by using a simple and accurate high-performance liquid chromatography (HPLC) method using photodiode-array detection (PDA) and aminosalicylic acid, as internal standard. Linearity of this method was kept from 0.01 to 100 mg of trimesic acid per liter of urine and from 0.05 to 5.00 wt% of trimesic acid per tissue weight. The limit of detection of the method was 0.01 μg per injection. This method was finally applied to analyze and quantify the amount of trimesic acid in rat urine and tissue samples at the different stages of degradation of MIL-100(Fe).

1. Introduction

Metal–organic frameworks (MOFs) are the latest class of porous solids and are highly tuneable hybrid materials built up from inorganic sub-units and organic polytopic ligands (carboxylates, phosphonates, ...), exhibiting a high and well-defined porosity [1–4]. They possess many interesting features that make them candidates for applications such as chemical sensing, gas storage, catalysis or separation [5]. Recently, their potential use in biomedicine has emerged [6], including therapeutics, controlled release of drugs, [7] biological gases [8], and diagnosis [9]. Several MOFs evaluated so far for biomedical applications are based on trimesic acid (1,3,5-benzene tricarboxylic acid). For instance, nitric oxide gas (antiplatelet aggregation agent, vasodilator and antibiotic) was loaded into a porous copper trimesate [10], leading to a complete inhibition of the platelet aggregation [8,11]. In addition, gadolinium(III) trimesate nanoparticles as contrast agents for Magnetic Resonance Imaging (MRI) have exhibited extraordinarily large longitudinal *r*1 and transversal *r*2 relaxivities due to the presence of a very high content of paramagnetic Gd^{3+} centres within each particle [12] while the less toxic silica coated manganese (II) trimesate nanoMOFs also exhibited very high *in vivo r*1 relaxivities [13]. Finally, non-toxic and biocompatible nanoparticles of the mesoporous iron(III) carboxylate MIL-100(Fe) [14], have combined interesting imaging properties, adapted for *in vivo* use, a very high loading capacity of several challenging drugs (antitumoral and antiretroviral) and a controlled release of these active therapeutic molecules under simulated physiological conditions [15].

Trimesic acid has also been used in other biological domains, such as the larval fish feed by the preparation of food microparticles through internal gelation of Ca–alginate [16], the crystallization of proteins via the crosslinking of the human hemoglobin between the Lysβ82 residues [17] and, more recently, the preparation of peptide dendrimers and hyperbranched polyamides for medical and biological applications such as catalysis, separation (chromatography) and encapsulation [18].

Based on its interest for bioapplications, there is thus a need to extract and quantify trimesic acid from biological media. However, to the best of our knowledge, no method for its extraction and dosage in biological samples has been reported so far. Therefore, we describe here an easy extraction procedure associated to a simple, sensitive and reproducible high-performance liquid chromatographic (HPLC) method to determine trimesic acid concentration in different biological samples such as liver, spleen and urine.

2. Experimental

2.1. Reagents

HPLC grade methanol, trimesic acid and aminosalicylic acid were purchased from VWR (France) and Sigma Aldrich (France),

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respectively. Sodium dodecyl sulfate (SDS), sodium hydrogen phosphate heptahydrated ($Na_2HPO_4{\cdot}7H_2O$), sodium dihydrogen phosphate dihydrated ($NaH_2PO_4{\cdot}2H_2O$) and orthophosphoric acid were obtained from Sigma Aldrich (France).

Standard solutions of trimesic acid (denoted S; $S_1 = 100\,mg\,L^{-1}$ and $S_2 = 10\,mg\,L^{-1}$) and standard solutions of aminosalicylic acid used as internal standard (denoted IS; $IS_1 = 200\,mg\,L^{-1}$ and $IS_2 = 20\,mg\,L^{-1}$), were prepared in methanol and stored at 4 °C in the dark. The phosphate buffer (0.04 M) was prepared in ultra pure water by mixing sodium hydrogen phosphate heptahydrated and sodium dihydrogen phosphate dihydrated. The pH was adjusted to 2.5 or 5 by adding some drops of orthophosphoric acid.

2.2. Liquid chromatography conditions

UV–visible spectra of trimesic acid and aminosalicylic acid were collected using standard solutions S_2 and IS_2 in a Shimadzu UV-160 A spectrophotometer. Maximum absorption for trimesic acid and aminosalicylic acid was 215 nm and 320 nm.

The delivered trimesic acid concentration was determined using a RP-HPLC system (Reversed phase liquid chromatography) equipped with a Waters Alliance C2695 separations module (Waters, Milford, MA, USA), a photodiode array detector (PDA) Waters E2998 and controlled by Empower software. The analytical column was a RP-C_{18} 5 μm Sunfire 150 × 4.6 mm I.D. (Waters) protected by HPLC cartridge precolumn C_{18} 5 μm Sunfire 20 × 4.6 mm I.D. (Waters). The mobile phase consisted in a solution of methanol in phosphate buffer of 0.04 M concentration, pH = 2.5. The mobile phase composition was modified to optimise the separation between trimesic acid and the internal standard, resulting in a better determination of trimesic acid in biological tissues (liver and spleen), which consists in a linear gradient from 5:95 to 50:50 (v/v) methanol–phosphate pH 2.5 (0–4 min) followed by an isocratic mode 50:50 (v/v) methanol–phosphate pH 2.5 (4–10 min). The flow rate was fixed to 0.8 mL min and the injection volume was 50 μL. In the case of urine samples, the applied conditions were a linear gradient from 10:90 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 (4–7 min). The flow rate was 1 mL min and the injection volume was 50 μL. Finally, washing and reconditioning of the column was done for 20 min. In all cases the column was operated at 37 °C and both the trimesic acid and the internal standard were monitored and quantified at 215 nm.

2.3. Animal studies

All experimental procedures were reviewed and approved by the Animal Experimentation Ethics Committee of the Faculty of Pharmacy of Paris-Sud 11 University, France. The study was performed on female Wistar rats (4-weeks-old; body weight 100–120 g) obtained from the central animal care facilities, Janvier R Centre d'Elevage, France. Rats were housed in individual metabolic cages and maintained in at 22–25 °C and 20% RH (relative humidity) with a 12 h light/dark cycle. Water and food were available during the hole of the experiment.

In order to determine the biodistribution of the trimesic acid in liver and spleen as well as its excretion in urine, 220 mg/kg of nanoparticles of MIL-100(Fe), denoted MIL-100_nano [15], were suspended in a 0.5 mL aqueous solution of glucose 10% and intravenously perfused through the jugular vein under isofluorane anesthesia. Six rats (denoted MIL-100) were used, according to a previously described procedure [15]. Likewise, a control group (named Glu group) was treated under the same conditions using 0.5 mL of glucose solution 10% [15]. 24 h after treatment, all animals were sacrificed under isofluorane anesthesia. Spleens and livers were extracted and washed with a NaCl 0.9% solution and stored at −20 °C until HPLC analysis. Urines samples were collected in 10 mL tube, then centrifuged (8000 g/15 min) and stored at −20 °C after the addition 0.1 mL of H_2SO_4 0.01 M per mL.

2.4. Calibration procedure

For liver and spleen samples, aliquots of 0.00, 0.25, 0.5, 1.0 or 2.0 mL of the trimesic acid standard S_1 (100 mg/mL) were placed with 1.5 mL of IS_1 in five 10 mL glass tubes. For urine samples, aliquots of 0.00, 0.25, 0.5, 1.0 or 2.0 mL of the trimesic acid standard S_2 (10 mg/mL) were placed with 0.1 mL of IS_1 in five 10 mL glass tubes.

2.5. Determination of trimesic 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 0.1 M SDS and 0.1 mL of IS_1 in 10 mL glass tubes. After homogenisation using a dispersing ultra-turrax, 1 mL of phosphate buffer (0.04 M, pH = 2.5) was added. The mixture was shaked and sonicated for 5 min. Then, 4 mL of methanol were added, 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 0.2 μm sterile syringe filter. In order to avoid an eventual partial extraction of the trimesic acid, the pellet was treated again under the same conditions. Then, the supernatant of each extraction was evaporated under a stream of nitrogen 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. Determination of trimesic acid in urine

A sample of 0.5 mL was centrifuged at 8000 × *g* for 10 min; the supernatant was filtered through 0.2 μm sterile syringe filter into an Eppendorf tube and diluted by 2 in phosphate buffer at pH = 5. In a 5 mL glass tube containing 0.5 mL of the diluted urine, 0.1 mL of IS_2 was added. After shaking for 1 min, 4 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 the mobile phase, right before the injection in the HPLC system.

2.7. Validation of the method

For liver and spleen tissues, linearity accuracy and recovery of the method were studied by spiking homogenised samples collected from control animals (1 g of liver taken from the right lobe or 0.1 g of spleen taken from right extremity + 0.5 mL of 0.1 M SDS), with 0.1 mL of trimesic standard solution S_1 and 0.1 mL of IS_1.

Likewise, for urine, linearity accuracy and recovery of the method were studied by spiking samples collected from control animals. Indeed, 0.5 mL urine samples were centrifuged at 8000 × *g* for 10 min. Then, the supernatants were filtered through a 0.2 μm sterile syringe filter into an Eppendorf tube and diluted by a 2 factor using a phosphate buffer at pH = 5.

A 0.5 mL of the diluted urine was spiked with 0.1 mL of trimesic standard solution S_2 and 0.1 mL of IS_2.

To check the specificity of the method, the peak purity of trimesic acid was investigated using the photodiode array detector and the retention time.

3. Results

The chromatograms obtained by the HPLC-PDA method are shown in Fig. 1. The identity of the trimesic acid (peak 2) was ascertained by its characteristic UV–vis spectra with a maximum

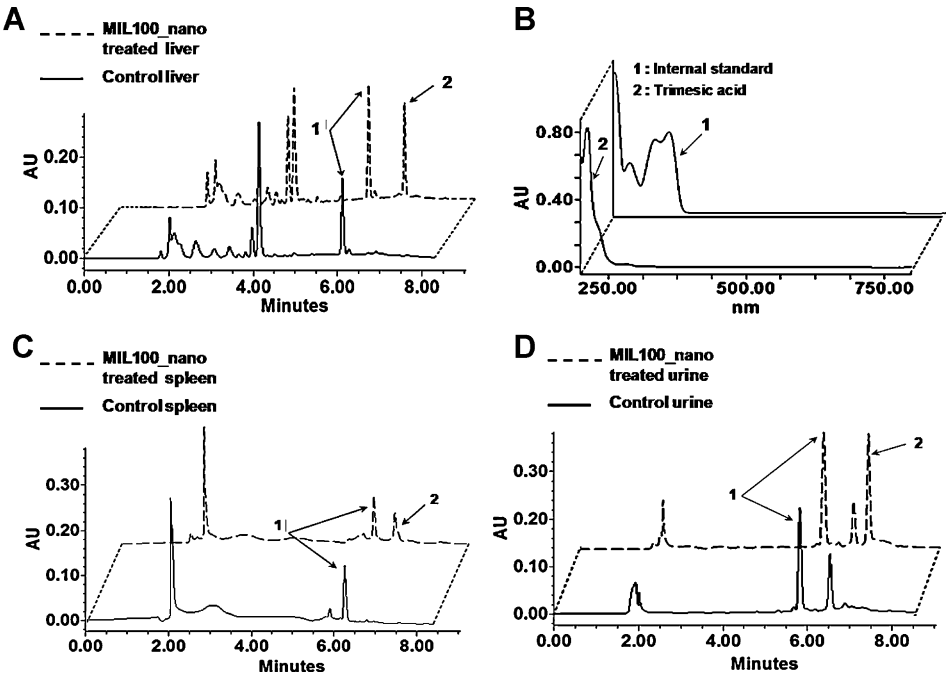

Fig. 1. Chromatograms of extracts of MIL-100_nano treated and control liver (A), spleen (C) and urine (D). (B) = UV–vis spectrum of internal standard (1) and trimesic acid (2) obtained by photodiode-array detection at 215 nm (chromatographic conditions for tissues: mobile phase: methanol in phosphate buffer of 0.04 M, pH = 2.5 using a linear gradient from 5:95 to 50:50 (v/v) methanol–phosphate pH 2.5 (0–4 min) followed by an isocratic mode 50:50 (v/v) methanol–phosphate pH 2.5 (4–10 min). Column temperature = 37 °C. Flow rate = 0.8 mL min^{-1}. Injection volume = 50 μL; chromatographic conditions for urines: mobile phase: methanol in phosphate buffer of 0.04 M, pH = 2.5 using a linear gradient from 10:90 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 (4–7 min). Flow rate was 1 mL min. Injection volume = 50 μL. Column temperature = 37 °C).

wavelength at 212.5 nm (Fig. 1B-2). Chromatograms profiles of liver (A), spleen (C) and urine (D) extract obtained from the control and the MIL100_nano treated rats, did not exhibit any peak interfering with IS (peak 1) at retention time 6.10 min or trimesic acid (peak 2) at retention time 7.25 min. The limit of detection of the method (signal/noise ratio = 3) was 0.01 μg of HPLC injected trimesic acid.

Both sensitivity and specificity of PDA mode were sufficient to determine the concentration of trimesic acid in tissues and urine. For liver and spleen samples, the linearity of the PDA method was confirmed from 0.05 to 5.00 wt% of trimesic acid, expressed as the % of the tissue weight ($y = 6.285x + 0.0146$, $r = 0.997$, $n = 6$ for livers, and $y = 6.154x + 0.009$, $r = 0.994$, $n = 6$ for spleens, where y is the area ratio trimesic acid/IS, x is the concentration of the trimesic acid and n is the number of samples). For urine, the linearity was checked for two concentration ranges: (i) from 0.01 to 1 mg L^{-1} ($y = 6.0203x + 0.537$, $r = 0.999$, $n = 6$) and (ii) from 10 to 100 mg L^{-1} ($y = 6.010x + 0.337$, $r = 0.999$, $n = 6$) where y is the trimesic acid/IS peak area ratio, x is the concentration of the trimesic acid in urine and n is the number of samples. The accuracy of the PDA method is shown in Table 1. Thus, relative standard deviation (RSD) did not exceed 5.2%.

The recovery of trimesic acid was determined by spiking samples collected from control animals at two concentrations (0.01 and 100 mg L^{-1} for urine; 0.05 and 5.00 wt% for tissues), comparing trimesic acid peak areas of biological samples issued from control and MIL-100_nano treated animals. For lower and upper values, extraction yields of trimesic acid ($n = 6$ for both samples) were 92 and 95 wt% for liver, 93 and 97 wt% for spleen and 96 and 98 wt% for urine, respectively (see Table 1). Under similar conditions, the recovery yields of the IS were 97, 96 and 98 wt% for liver, spleen and urine, respectively. In all cases, extraction yield of trimesic acid, estimated by comparison with IS, exceeded 96 wt% for both upper and lower values.

Finally, some preliminary results of the trimesic acid biodistribution issued from the MIL-100_nano administered intravenously to rats are shown in Fig. 2. It could be calculated that 24 h after injection, trimesic acid concentration of the treated rats ranged from 0.91 to 1.6 mg/g (mean=1.24 mg/g), 1.13 to 2.63 mg/g (mean = 2.10 mg/g) and from 0.022 to 0.042 mg/mL (mean = 0.032 mg/g), for liver, spleen and urine respectively.

4. Discussion

The proposed gradient elution method allowed an efficient separation of the internal standard as well as of the trimesic acid. This sensitive and specific simple liquid chromatographic method enabled to identify and quantify trimesic acid from all the studied biological samples (liver, spleen and urine), keeping the linearity in a large range of concentrations. However, biological sample preparation prior to the injection into the chromatograph is an important issue. Prior to HPLC analysis of trimesic acid extracted from liver and spleen samples, several steps were needed. For an accurate

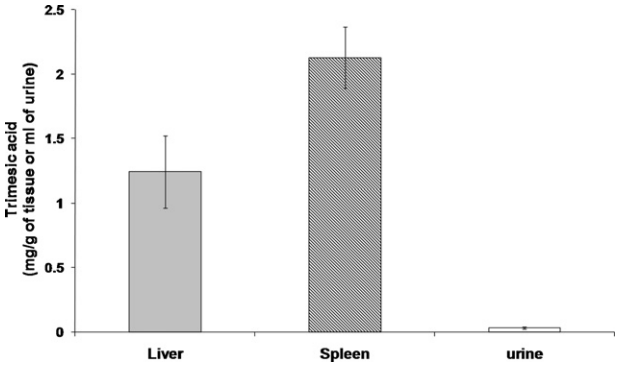

Fig. 2. Trimesic acid concentrations in liver (1.24 mg $g^{-1} \pm 0.28$), spleen (2.10 mg $g^{-1} \pm 0.53$) and urine (0.03 mg $mL^{-1} \pm 0.01$) 24 h after MIL100_nano administration to rats ($n = 6$); administered dose: 220 mg/kg of body weight.

Table 1
Precision of the method.

Spiked samples	Added concentration	Accuracy – repeatability			
		Concentration determined within-day ($n = 6$)		Concentration determined between days ($n = 6$)	
		Mean	RSD (%)	Mean	RSD (%)
Liver 1	0.050%	0.051%	4.8	0.049%	5.2
Liver 2	5.000%	5.020%	2.5	4.970%	3.2
Spleen 1	0.050%	0.052%	2.2	0.051%	4.5
Spleen 2	5.000%	5.008%	3.5	5.009%	3.6
Urine 1	0.100 mg L^{-1}	0.102 mg L^{-1}	4.5	0.098%	4.1
Urine 2	1.000 mg L^{-1}	1.015 mg L^{-1}	3.1	0.998%	4.2
Urine 3	100.000 mg L^{-1}	100.205 mg L^{-1}	1.2	100.200%	3.6

quantification, lysis of cellular membranes with detergents such as SDS was required in order to release the trimesic acid fraction that might be trapped in the cells. In addition, although trimesic acid is soluble in methanol (1.5 mg/mL at 25 °C), a prolonged contact time under stirring (around 1 h) was required to complete full extraction of the acid, which was then easily separated from the co-extracted lipophilic substances, generally eluted with the solvent front.

For the urine samples, extraction of trimesic acid was easier due both to the higher solubility of its fully deprotonated form in slightly basic urine pH = 8 (p*K*a of trimesic acid: 3.12, 3.89 and 4.7) [19] and to the lower degree of complexity of urine matrix compared with liver and spleen tissues. Indeed, the initial treatment by centrifugation and filtration was efficient to remove impurities from urine samples. Thus, only 15 min of stirring was necessary to obtain a significant liquid–liquid extraction of trimesic acid in urine.

Finally, this method could be successfully applied to the determination of the trimesic acid on rats administered intravenously with nanoparticles made of iron trimesate MIL-100 (Fig. 2). Thus, an interindividual variation of around 3% for urine and spleen, and around 25% for the liver was observed, due to the different kinetics of biodegradation processes of nanoparticles from different rats. Nevertheless, these preliminary results showed that MIL100 nanoparticles were rapidly captured by the reticulo-endothelial organs after intravenous administration. Thus, trimesic acid extraction and determination by this HPLC-PDA method paves the way for further biodistribution studies required prior to use MIL100_nano systems in drug delivery and/or imaging applications.

5. Conclusion

A simple and accurate liquid–liquid extraction, separation and quantification method of trimesic acid, a constitutive organic linker of MOFs, has been successfully developed for liver, spleen and urine analysis. After liquid–liquid extraction with a recovery yield exceeding 92 wt%, determination of trimesic acid was performed by an HPLC method using PDA detection and aminosalicylic acid as internal standard. Both, linearity, kept from 0.01 to 100 mg of trimesic acid per liter of urine and from 0.05 to 5.00 wt% of trimesic acid per tissue weight, and limit of detection (0.01 μg per injection) were in agreement with the high accuracy of the method. This method will allow an in-depth future biopharmaceutical analysis of MOFs nanoparticulate systems based on trimesate linker.

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