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A simple HPLC-ESI-MS method for the direct determination of ten pteridinic biomarkers in human urine

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ABSTRACT

Pteridines are important biomarkers metabolites related to several biochemical pathways such as activation of the cell-mediated immune system, biosynthesis of neurotransmitters, etc. The level of pteridinic compounds in urine is considered as an important clinic criterion. In this work, a new liquid chromatography-mass spectrometry (LC-MS) method is proposed to determine several pteridinic biomarkers in urine samples using 6-methylpterin as internal standard (I.S.). Matrix effect was evaluated and several dilutions of urine were tested in order to study the evolution of signal suppression. Sample preparation was limited to 10-fold dilution of the filtered urine followed by injection onto a reversed-phase column. The signal was recorded in selected ion monitoring mode. The lowest limit of detection was found for pterin (values ranged from 1.70 to 3.88 ng mL^{-1}) whereas the highest limit was for xanthopterin (values ranged from 10.5 to 49.9 ng mL^{-1}) for healthy volunteers between 17 and 51 years old.

1. Introduction

While the clinical symptoms of a disease are endpoints in themselves, they are not suitable, in many cases, for early detection and, therefore, prevention of disease. Currently, using first level biomarkers may be proven to relate directly to functional changes, and ultimately disease [1].

Non-conjugated folates (pteridines) belong to a heterocyclic family formed by a bicyclic pyrimidine–pyrazine moiety that occurs in a wide range of living systems and participates in relevant biological functions [2]. Pteridines exist in different oxidation states and as consequence they are divided into two classes: oxidized or aromatic pteridines, such as biopterin (BIO), neopterin (NEO) between others, and reduced pteridines, dihydropteridines and tetrahydropteridines, which are the ones mostly excreted in biological fluids [3]. Within the latter group, 7,8-dihydropteridines and 5,6,7,8-tetrahydropteridines are the most important derivatives because of their biological activity, i.e. 7,8-dihydroneopterin (NH2) is excreted during the oxidative burst of stimulated macrophages. 7,8-dihydrobiopterin (BH2) and 5,6,7,8-tetrahydrobiopterin (BH4) participate in the metabolism of aminoacids and play a vital role in various biochemical pathways [4].

Pteridines and its derivatives are important cofactors in the process of cell metabolism. The urinary excretion of these compounds increases as a result of several disorders such as viral infection and different types of cancer [5,6]. Hence, the level of these compounds in urine, plasma and cerebrospinal fluid is established as an important clinical criterion. Although NEO has been classically considered as the most important pteridine as cancer marker, ratios of other pteridines are also used in clinical laboratories for cancer and precancer screening. Significant increase in the urinary excretion of xanthopterin (XAN), NEO and pterin (PT) [1], as well as significant decrease in isoxanthopterin (ISO) and NEO/BIO increment, was found in cancer patients [6]. It was reported that the mean NEO/BIO ratio for healthy individuals is 1.27 and for cancer patients is 2.59 [6] using CE analysis. On the other hand, an increase in the pterin-6-caboxylic acid (PT6C) excretion and a reduction in ISO were also reported by the same authors.

An increase of the NEO amount was also observed in children infected with mononucleosis [7]. NEO concentration was also found to be correlated with decline Alzheimer's disease patients [8].

It is known that alterations on the metabolic mechanism of BH4 give rise to different diseases, named hyperphenylalaninaemia (HPAs). The differential diagnostic of these HPAs is important to establish the adequate therapeutic protocol and is based on the measurement of pteridines and other metabolites excretion. Hence, the classical phenylketonuria produces increments in the urinary levels of NEO and BIO while the BH4 deficit produces a

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decrease in the NEO and BIO levels [9–11]. Thus, NEO/BIO ratio is also used as a diagnostic criterion.

The quantitative determination of pteridines is complicated because of their low concentration in body fluids, low solubility, their photosensitivity and their occurrence in three forms (tetrahydro-, dihydro-, and completely oxidized form). Traditionally, analysis of pteridines required their oxidation into their highly fluorescent aromatic forms. Under acidic conditions iodine treatment gives near complete (90–100%) conversion of dihydro- and tetrahydropteridines to their fully oxidized forms. However, under basic oxidation conditions [12], tetrahydropteridines are converted to 2-amino-4-hydroxypteridine (pterin) due to cleavage of the 6-side chain, whereas dihydropteridines are converted to their respective oxidized pterines. Hence, BH4, have been determined by differential methods, with the loss of security that this type of analysis involves [13].

In the recent bibliography, HPLC separation accomplished with fluorimetric detection is widely applied in order to obtain adequate detection limits in biological fluids analysis [7,14–16]. Although liquid chromatography-mass spectrometry (LC-MS) is a technique widely applied in the analysis of biological fluids, it has been rarely used in the pteridinic markers analysis. Hydroxymethylpterin was analyzed in several samples by comparing its retention time, mass, and fragmentation pattern with that of a known standard [17]. Stea and coworkers report a method for the derivatization and GC–MS analysis of 6-hydroxymethylpterin [18]. NEO and BIO have been determined in rats and human plasma, cell extracts and tissue homogenates by LC-MS/MS using a QTRAP triple quadrupole mass spectrometer [19]. Recently, it has been reported the evaluation of hydrophilic interaction liquid chromatography (HILIC) in the analysis of polar pteridines using MS detection, but it has not been implemented to any real sample [20]. As far as we know, this is the first contribution on which a HPLC-ESI-MS method has been developed to determine several pteridinic biomarkers (chemical structures are shown in Fig. 1) in human urine in the oxidation level they are excreted. Sample preparation was limited to 10-fold dilution of the urine into the internal standard solution followed by injection. The method has been validated in terms of linearity, limits of detection and quantification and repeatability, and it has been applied to the analysis of urine from seven healthy adults. The amount of pteridines was reported as a ratio of pteridine to creatinine in order to assure that the pteridine levels represent the physiological concentration. Moreover, NEO/BIO ratios were calculated. Thus this method allows easy determination without prior stages of sample treatment.

2. Material and methods

2.1. Chemicals and reagents

Creatinine (CREA), ditiotreitol (DTT), NEO, BIO, PT6C, PT, monapterin (MON), ISO, XAN, 6-hydroxymethylpterin (6HMPT) and NH2 were purchased from Sigma (Sigma-Aldrich Química, S.A., Madrid). BH2 and 6-methylpterin (6MPT) were from Schircks Laboratories (Jona, Switzerland). Picric acid and sodium dodecyl sulfate (SDS) were from (Sigma-Aldrich Química, S.A., Madrid). Phosphoric acid and methanol were purchased from Scharlau (Scharlau, Barcelona). Formic acid and ammonium formate were from Sigma (Sigma-Aldrich Química, S.A., Madrid). Acetonitrile (HPLC grade) was purchased from Merck (Madrid, Spain). Ultrapure water was obtained using a Milli-Q system (Waters Millipore, Milford, MA, USA).

2.2. Sample preparation

2.2.1. Standard preparation

Stock standard solutions (15 μg mL^{-1}) were prepared by exact weighting of each pteridine and hydropteridine, dissolution with alkalinized ultrapure grade water and later neutralization with hydrochloric acid. BH2 and NH2 standard solutions also contain 0.1% DTT to minimize the spontaneous oxidation of the reduced species due to environmental oxygen [7]. Exposure to direct sunlight was avoided. Pteridine standard solutions were stored at −18 °C in the dark and they were stable for at least 5 days. NH2 and BH2 stock standard solutions were prepared daily. CREA standard solution (1.0 mg mL^{-1}) was prepared in ultrapure water and stored at 4 °C.

A standard working mixture solution containing 1.5 μg mL^{-1} of each pteridine was prepared by dilution of the stock standard solutions with ultrapure water. A standard working mixture solution containing both hydropteridines, NH2 and BH2, (1.5 μg mL^{-1}) was prepared by dilution with ultrapure water containing 0.1% DTT. Other solutions were prepared via serial dilutions (and always containing 0.1% DTT) and used in the generation of the calibration curves.

2.2.2. Urine samples

The first morning urine samples from seven healthy adults (5 women and 2 men; age range 17–51 years) were collected and stored, either in the refrigerator (4 °C) or freezer (−18 °C), depending on the time from collection to analysis. Prior to analysis, the samples were removed from cold storage and brought to room temperature. Urine samples were collected from volunteers that kindly consented to do the analysis.

Three milliliters of urine were filtered through 0.2 μm membrane nylon filters. 100 μL of filtered urine were pipette into an autosampler vial containing 20 μL of the 1.25 μg mL^{-1} internal standard solution, 0.1% DTT, variable volumes of the standard working mixtures of pteridines and dihydropteridines (1.5 μg mL^{-1}) and diluted to 1 mL with ultrapure water. Each analysis was realized in triplicate.

2.3. Instrumentation and software

The chromatographic studies were performed on an Agilent Model 1100 LC instrument (Agilent Technologies, Palo Alto, CA, USA), equipped with degasser, quaternary pump, column oven, autosampler Agilent 1290 infinity thermostated at 5 °C and the CHEMSTATION software package to control the instrument, data acquisition and data analysis. The analytical column employed was a Zorbax Eclipse XDB-C18, 250 mm × 4.6 mm and 5 μm particle size (Agilent Technologies). The column temperature was set at 22 °C. The mobile phases were high-purity water with 0.1% formic acid and 2% acetonitrile (A) and acetonitrile with 0.1% formic acid (B). The gradient program was as follows: 100% of mobile phase A during 8 min followed by a linear gradient to 80 % A in 8 min and then constant for another 4 min. A 10 min of post-run time back to the initial mobile phase composition was used after each analysis. Mobile phases were filtered through a 0.22 μm membrane nylon filter and degassed by ultrasonication before use. The flow rate was set constant at 0.6 mL min^{-1} and the injection volume was 20 μL.

Detection was performed with a mass spectrometer Agilent Technologies single quadrupole 6120 equipped with an electrospray interface operated in the positive ionization mode. Nitrogen was used as the nebulizer gas. Optimization of the compounds was performed by flow injection analysis injecting individual standard solutions directly into the source. Initially, both positive

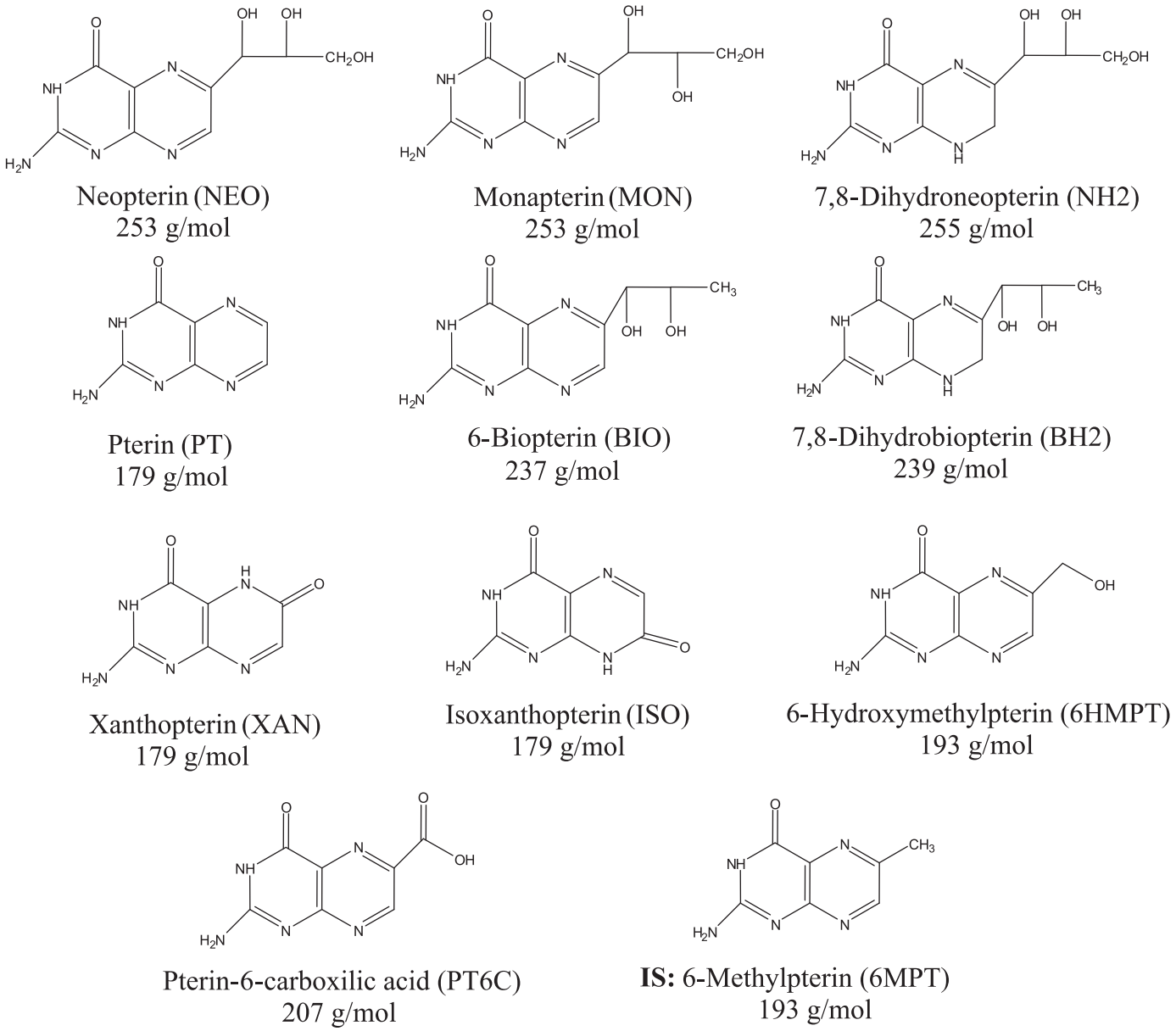

Fig. 1. Structures and molecular masses of pteridinic markers studied.

and negative ionization modes were investigated and positive polarity was preferred due to its higher sensitivity. Single ion monitoring (SIM) was selected as operation mode using the target ion $[M+H]^+$ for all studied compounds.

Varian spectrophotometer, model Cary 50 Bio, equipped with a Xenon lamp was used for the acquisition of the photometric measurements and kinetic data used in the creatinine determination.

Calibration curves and analytical figures of merit were performed by means of the ACOC program, developed in our research group, in MatLab code [21].

2.4. Matrix effect

For each compound, the slopes obtained in the calibration with matrix-matched standards were compared with those obtained with solvent-based standards, calculating urine/water slope ratios at different urine dilution levels. Specifically, five different calibration curves were prepared for each compound, one in solvent, and four in urine using dilution factors of 5, 10, 20 and 50. Each calibration curve comprised five concentration points in triplicate within the range of 20–200 ng mL^{-1} for all pteridines except XAN, for which the range 40–400 ng mL^{-1} was employed due to its lower sensitivity.

For the I.S., the matrix effect was evaluated by comparing the peak areas of 6MPT at the concentration level selected for the study (25 ng mL^{-1}) in urine at different dilutions (5, 10, 20 and 50) and in water. For each urine dilution, 10 identical spiked samples with the I.S. were prepared and the average peak area was used for calculation.

2.5. Method validation

For quantification purposes, five matrix-matched standards of concentrations between 0 and 100 ng mL^{-1} were prepared by spiking the urine samples with appropriate volumes of working standard solutions (corresponding to a concentration range of 0–1.0 μg mL^{-1} referred to undiluted urine). For XAN, concentration ranged up to 400 ng mL^{-1} due to its lower sensitivity (corresponding to a concentration ranged up to 4.0 μg mL^{-1} in undiluted urine). Three independent experiments were carried

out for each concentration level. Pteridine/I.S. peak area ratios of the extracted ion chromatograms (EICs) were used for quantification purposes in order to improve the precision of quantitative analysis.

The limit of detection (LOD) was calculated according to the Long & Winefordner criterion [22]. Limit of quantification (LOQ) of the method was defined as 10/3 times LOD. Precision was assessed as the percentage relative standard deviation (% RSD) of both repeatability within-day ($n=6$) and between-day ($n=5$) for all compounds in a pool of 10-fold diluted urine spiked with a pteridine standard mixture at a concentration range within the calibration range.

2.6. Determination of creatinine

CREA was determined in urine samples with a kinetic-photometric method based on the Jaffe reaction [23]. It was treated with an alkaline picrate solution to yield a bright orange–red complex which was determined photometrically at 510 nm. The formation rate of the complex measured through the increase of absorbance in a prefixed interval of time was proportional to the concentration of CREA in the sample [24–26].

The reagent used to determine CREA in urine consisted on 25 mM of picric acid prepared in 300 mM phosphate buffer (pH 12.1) and 2 g L^{-1} SDS (to avoid protein precipitation). To conduct the kinetic-photometric method, 2 mL of picric acid solution was transferred to the photometric cell which was thermostated at 37 °C. Then, 200 μL of 10-fold diluted urine was added and then, the evolution of the absorbance with time at 510 nm was scanned during 2 minutes. The reaction rate was measured as the tangent in the linear part of the kinetic curve between 0.3 and 1.5 min. Each sample was assayed in triplicate.

3. Results and discussion

3.1. Method development

3.1.1. Optimization of the LC separation

A pteridines mixture in urine 10-fold diluted was employed to realize the optimization of the LC separation method. During this study, acetonitrile and methanol were evaluated as organic modifiers of the mobile phase, as well as the volatile additives formic acid and ammonium formate. The best chromatographic peak shapes and signal-to-noise (S/N) ratios of the analytes were obtained by the use of water/acetonitrile mixture with 0.1% formic acid.

A gradient elution was necessary because of the wide capacity factor's range of the pteridines in study. Firstly, the initial percentage of acetonitrile in the mobile phase A was evaluated in order to optimize the separation of the early eluting pteridines. In Fig. 2, the separation of the isomers NEO and MON at different water/acetonitrile percentage is shown. As it can be observed, an initial percentage of acetonitrile of 5% results in strong overlapping of both isomers preventing its quantification. However, when the mobile phase contained 2% of acetonitrile, both peaks are well resolved. Therefore, an initial water (with 0.1% of formic acid)/acetonitrile ratio of 98/2 (v/v) (mobile phase A) was selected as adequate to resolve the most polar pteridines. Then, different elution gradients, using increasing percentages of acetonitrile with 0.1% formic acid (mobile phase B), were tested in order to resolve the less polar pteridines and to achieve the separation of PT6C from an unknown interference with the same m/z, which was the more restricting parameter to shorten the chromatographic time. Finally, the gradient that provided the best separation and the greater baseline stability was as follows: 100% of

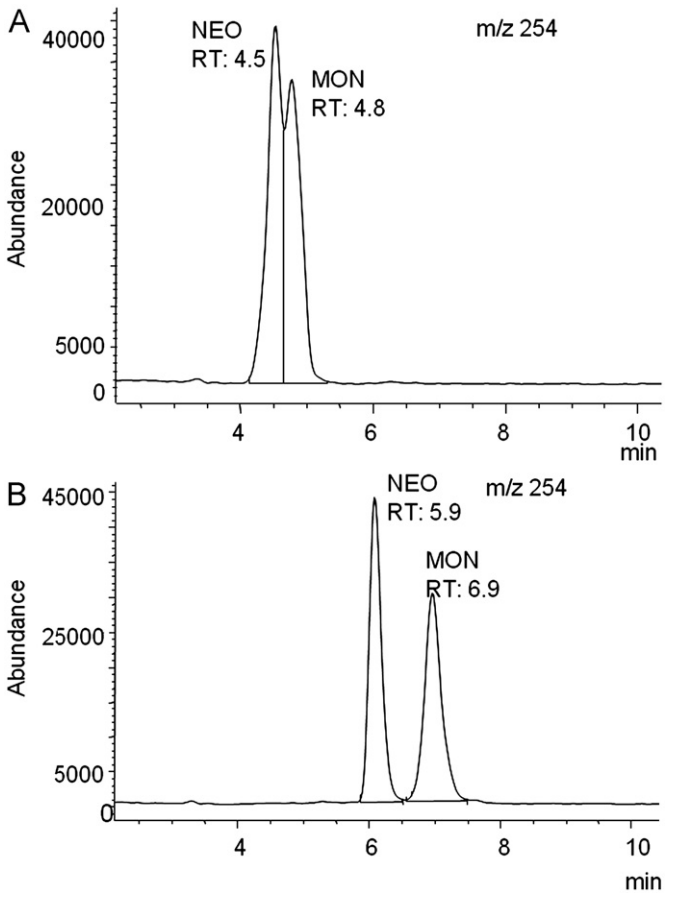

Fig. 2. Chromatograms obtained from isomers NEO and MON mixture using water with 0.1% formic acid and 5% acetonitrile (A) and water with 0.1% formic acid and 2% acetonitrile (B) in isocratic mode.

mobile phase A during 8 min followed by a linear gradient to 80% A in 8 min and then constant for another 4 min. A 10 min of post-run time back to the initial mobile phase composition was used after each analysis. Fig. 3 shows the total ion chromatogram (TIC) (including the gradient mode optimized) and the EICs obtained from the analysis of a pteridines mixture in urine 10-fold diluted. As it can be observed, good resolution was achieved for both isomeric pteridines NEO-MON and XAN-ISO with the same m/z.

3.1.2. MS optimization

Flow injection analysis (FIA) of individual standard solutions at a concentration of 300 ng mL^{-1} was performed to optimize the main instrumental parameters (fragmentor and capillary voltage, nebulizer pressure, nitrogen flow rate and temperature) in ESI positive mode. Fragmentor voltage was optimized in the first place and mass spectra obtained in the full scan mode at different fragmentor voltages were investigated. Both positive and negative ionization modes were investigated in order to obtain the highest sensitivity for the analytes. The voltage value was studied in the range of 50 V–325 V. The results showed that the base peak (the highest ion peak in the mass spectrum, which can be selected as the target ions of the analytes) in the mass spectra was the protonated molecular ion, $[M+H]^+$. Fragmentor voltages of 150 V and higher led to extensive fragmentation even of the reference masses. The relative intensities of the protonated molecular ion of PT, 6HMPT and PT6C increased up to 75 V and decreased for higher voltages. However, the rest of analytes showed the highest abundance of the base peak with a fragmentor voltage of 100 V.

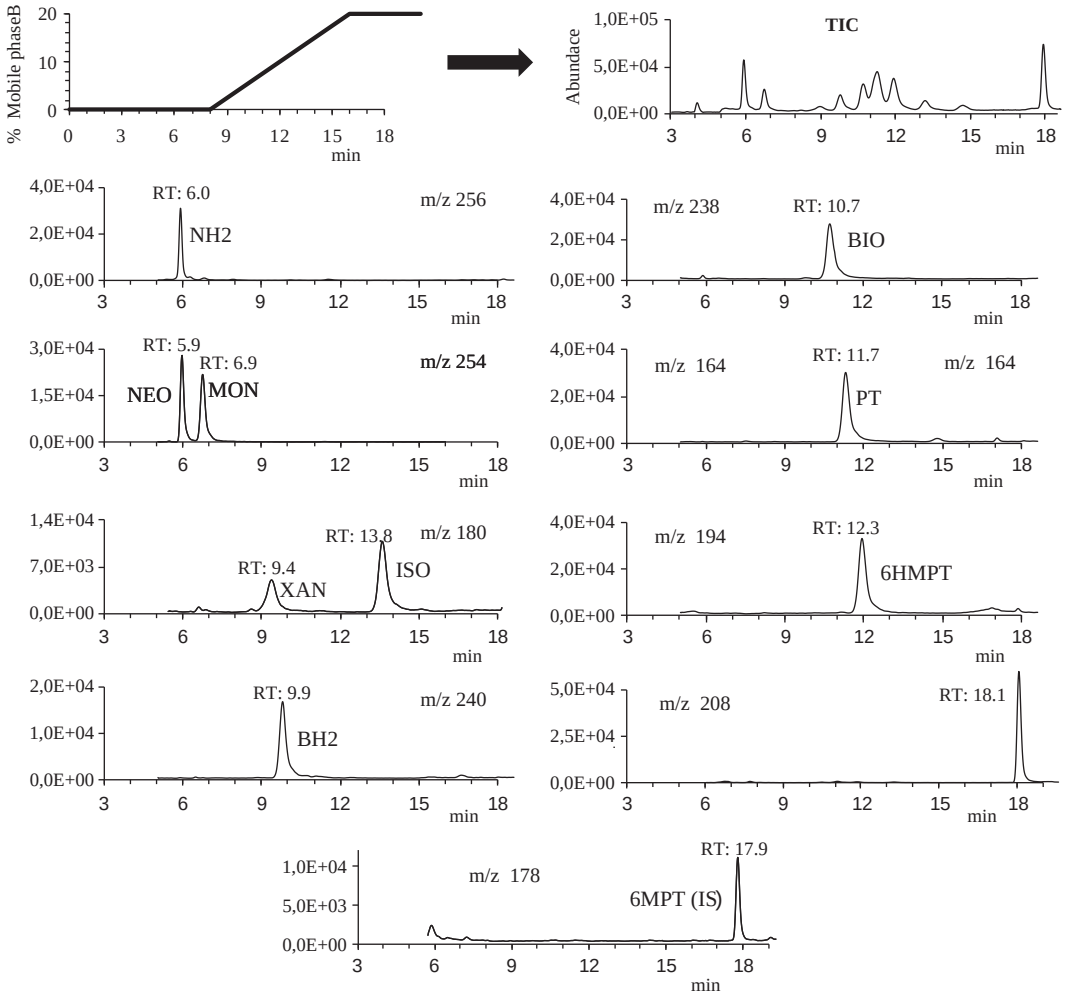

Fig. 3. TIC and EICs obtained from the analysis of pteridines and hydropteridines mixture in urine (10-fold diluted) with the optimized separation method.

A compromise value of 100 V was chosen for further experiments. The other instrumental parameters: drying gas flow rate, nebulizer pressure, capillary voltage and gas temperature, were evaluated in the SIM mode and optimized to obtain the highest intensity of the protonated molecular ion. Finally and in terms of sensitivity, the instrumental variables were set as follows: nebulizer pressure: 40 psig; drying gas: 10 L/min; gas temperature: 300 °C; capillary voltage: 4000 V; fragmentor voltage: 100 V.

3.2. Matrix effect

One significant drawback, especially when using electrospray ionization, is that the presence of matrix components may affect the ionization of the target analytes producing an ion formation suppression effect [27]. In these cases, the precision and accuracy of the method should be assessed using biofluids from different sources (subjects) [28]. To evaluate the extension of this effect, a pool of urine from different individuals at different dilution factors (5, 10, 20 and 50) was spiked with the standard mixture of pteridines at five different levels in triplicate and analyzed. A comparison between the slopes of calibration curves in water and in presence of urine for each analyte, was accomplished by applying the *F* and *t* statistical tests [29]. The F-test does not indicate significant differences, but the t-test shows $t_{cal} > t_{tab}$ for all analytes at the 95% confidence level, indicating that the calibration slopes were not comparables and matrix effect was present. The results obtained for the different dilutions are summarized in Table 1. Signal suppression was observed for all analytes in all urine samples evaluated. Although a softer matrix effect was found when 20-fold dilution of urine and higher

Table 1
Retention times and matrix effects in the analysis of pteridines in urine at different dilution factors.

Compound	t_R (min)	Calibration curve in wáter			% Matrix effect[a]			
					Dilution factor			
		Slope ± SD (mL ng^{-1})	$S_{y/x}$[b]	R^2	5	10	20	50
NEO	5.9	0.0261 ± 0.0008	0.0811	0.994	−65	−44	−40	−15
NH2	6.0	0.0179 ± 0.0006	0.0657	0.992	−57	−40	−35	−5
MON	6.9	0.0230 ± 0.0007	0.0730	0.994	−79	−33	−28	−14
XAN	9.4	0.0170 ± 0.0003	0.0742	0.992	−67	−44	nd	nd
BH2	9.9	0.0173 ± 0.0006	0.0600	0.993	−44	−31	−14	6
BIO	10.7	0.0269 ± 0.0009	0.0913	0.993	−63	−23	−14	−12
PT	11.7	0.0320 ± 0.0010	0.103	0.994	−79	−15	−10	−2
6HMPT	12.3	0.0235 ± 0.0008	0.0820	0.993	−66	−14	−10	−4
ISO	13.8	0.0118 ± 0.0005	0.0492	0.990	−76	−22	−13	−3
PT6C	18.1	0.0472 ± 0.0018	0.177	0.993	−71	−20	−15	−8

Note: nd = not detected; each calibration curve comprises five concentration levels in triplicate (20–200 ng mL^{-1} for all pteridinas except XAN: 40–400 ng mL^{-1}).

[a] % Matrix effect = 100 × (urine/water slope ratio) – 100.

[b] $S_{y/x}$ = Standard error of the estimate.

Table 2
Analytical parameters for the analysis of pteridines in presence of urine (dilution factor: 10) by the HPLC-ESI-MS proposed method.

		Woman (51)[a]	Man (51)[a]	Man (50)[a]	Woman (33)[a]	Woman (32)[a]	Woman (22)[a]	Woman (17)[a]
NEO	Slope ($\times 10^2$) (mL ng^{-1})	1.38 ± 0.04	1.95 ± 0.07	2.14 ± 0.07	1.38 ± 0.03	2.20 ± 0.03	1.87 ± 0.03	1.48 ± 0.04
	Intercept ($\times 10^2$)	40.9 ± 2.2	66.0 ± 3.3	45.0 ± 2.9	45.1 ± 1.5	36.6 ± 1.8	67.8 ± 1.6	70.8 ± 2.1
	Sy/x[b]	0.0537	0.0813	0.0698	0.0395	0.0492	0.0431	0.0491
	LOD[c]/LOQ[d] (ng mL^{-1})	6.89/22.9	7.75/25.8	5.74/19.1	5.03/16.7	3.06/10.2	4.24/14.1	7.92/26.4
NH2	Slope ($\times 10^2$) (mL ng^{-1})	1.56 ± 0.04	1.33 ± 0.05	2.00 ± 0.08	1.81 ± 0.05	2.45 ± 0.08	1.45 ± 0.06	1.21 ± 0.05
	Intercept ($\times 10^2$)	242.2 ± 2.0	238.1 ± 2.6	246.9 ± 3.6	395.9 ± 2.6	183.1 ± 6.2	268.6 ± 2.8	244.8 ± 0.4
	Sy/x[b]	0.0544	0.0677	0.0695	0.0710	0.1261	0.0700	0.0739
	LOD[c]/LOQ[d] (ng mL^{-1})	14.5/48.3	24.5/81.6	19.0/63.3	22.4/74.6	14.1/46.9	27.3/90.9	33.1/110
MON	Slope ($\times 10^2$) (mL ng^{-1})	1.10 ± 0.04	1.84 ± 0.05	1.78 ± 0.05	9.40 ± 0.02	1.79 ± 0.03	1.63 ± 0.03	1.35 ± 0.05
	Intercept ($\times 10^2$)							
	Sy/x[b]	0.0434	0.0607	0.0511	0.0247	0.0313	0.0329	0.0635
	LOD[c]/LOQ[d] (ng mL^{-1})	6.22/20.7	4.72/15.7	4.15/13.8	3.70/12.3	2.88/9.6	2.91/9.7	6.28/20.9
XAN	Slope ($\times 10^2$) (mL ng^{-1})	2.16 ± 0.08	1.09 ± 0.03	2.19 ± 0.13	1.38 ± 0.06	1.91 ± 0.08	1.68 ± 0.02	1.60 ± 0.06
	Intercept ($\times 10^2$)							
	Sy/x[b]	0.1581	0.0662	0.3999	0.1184	0.1485	0.0405	0.1823
	LOD[c]/LOQ[d] (ng mL^{-1})	32.3/108	24.9/82.9	49.9/166	37.7/125	34.0/113	10.5/35.0	31.2/104
BH2	Slope ($\times 10^2$) (mL ng^{-1})	2.61 ± 0.11	2.00 ± 0.07	2.07 ± 0.06	3.18 ± 0.15	4.00 ± 0.16	1.84 ± 0.05	1.95 ± 0.09
	Intercept ($\times 10^2$)	280.1 ± 5.8	101.8 ± 1.9	97.2 ± 3.1	589.8 ± 7.6	338.1+14.4	171.2 ± 1.8	645.7 ± 6.1
	Sy/x[b]	0.1605	0.0344	0.0787	0.2071	0.2915	0.0424	0.0915
	LOD[c]/LOQ[d] (ng mL^{-1})	20.9/69.6	7.37/21.4	9.75/32.5	31.3/104.2	20.03/66.7	9.05/30.1	55.5/184.8
BIO	Slope ($\times 10^2$) (mL ng^{-1})	2.03 ± 0.04	2.92 ± 0.11	2.44 ± 0.08	1.69 ± 0.07	2.56 ± 0.04	2.26 ± 0.07	1.91 ± 0.06
	Intercept ($\times 10^2$)	29.1 ± 1.9	80.9 ± 4.5	21.6 ± 3.4	42.3 ± 3.3	27.9 ± 2.1	35.8 ± 2.6	61.1 ± 2.9
	Sy/x[b]	0.0469	0.1084	0.0674	0.0804	0.0439	0.0698	0.0747
	LOD[c]/LOQ[d] (ng mL^{-1})	3.42/11.4	7.2/24.0	4.87/16.2	7.16/23.8	2.48/8.26	4.64/15.5	6.95/23.1
PT	Slope ($\times 10^2$) (mL ng^{-1})	1.95 ± 0.03	3.70 ± 0.08	2.43 ± 0.09	1.69 ± 0.05	3.49 ± 0.04	3.18 ± 0.04	2.12 ± 0.05
	Intercept ($\times 10^2$)	35.6 ± 2.0		25.3 ± 1.1	18.9 ± 2.3		26.6 ± 2.0	30.8 ± 1.8
	Sy/x[b]	0.0245	0.0867	0.0212	0.0630	0.0387	0.0559	0.0430
	LOD[c]/LOQ[d] (ng mL^{-1})	3.88/12.9	3.49/11.6	2.34/7.79	2.63/8.76	1.70/5.66	2.20/7.33	3.13/10.4
6HMPT	Slope ($\times 10^2$) (mL ng^{-1})	1.91 ± 0.05	1.83 ± 0.04	2.45 ± 0.09	1.79 ± 0.06	2.49 ± 0.06	2.15 ± 0.07	1.42 ± 0.06
	Intercept ($\times 10^2$)							
	Sy/x[b]	0.0648	0.0413	0.0928	0.0614	0.0674	0.0816	0.0593
	LOD[c]/LOQ[d] (ng mL^{-1})	5.23/17.4	3.31/11.0	5.46/18.2	4.94/16.5	3.82/12.7	5.49/18.3	6.59/21.9
ISO	Slope ($\times 10^2$) (mL ng^{-1})	1.89 ± 0.05	1.19 ± 0.03	1.01 ± 0.02	1.83 ± 0.04	1.55 ± 0.03	1.01 ± 0.02	0.10 ± 0.02
	Intercept ($\times 10^2$)	5.78 ± 2.59		3.00 ± 0.92	10.4 ± 1.9		10.1 ± 0.8	15.1 ± 0.9
	Sy/x[b]	0.0662	0.0219	0.0183	0.0511	0.0376	0.0150	0.0243
	LOD[c]/LOQ[d] (ng mL^{-1})	4.06/13.5	2.82/9.39	2.88/9.59	3.37/11.2	3.42/11.4	2.76/9.19	3.27/10.9
PT6C	Slope ($\times 10^2$) (mL ng^{-1})	2.90 ± 0.06	1.16 ± 0.05	1.76 ± 0.04	2.99 ± 0.08	3.61 ± 0.04	3.47 ± 0.05	3.27 ± 0.05
	Intercept ($\times 10^2$)							
	Sy/x[b]	0.0799	0.0481	0.0330	0.0395	0.0537	0.0521	0.0449
	LOD[c]/LOQ[d] (ng mL^{-1})	3.94/13.1	4.20/14.0	2.85/9.50	4.13/13.8	2.19/7.29	2.32/7.73	1.68/5.59

Note: Calibration range: 10–100 ng mL^{-1} for all pteridinas except XAN (150–400 ng mL^{-1}). Blank urines were also included in the calibration. Intercept values of the calibration curve equal or below cero are shaded.

[a] Age.
[b] Standard error of the estimate.
[c] Limit of detection according to the Long & Winefordner criterion (k=3) [24].
[d] LOQ = (10/3) LOD.

was employed, detectability of XAN was not assured in the studied range (40–400 ng mL^{-1}). When a dilution factor of 5 was evaluated, a strong matrix effect was found for all compounds ($\geq$44%). Therefore, a compromise dilution factor of 10 was selected in order to obtain the softest possible matrix effect assuring the detectability of all compounds at values reported in literature [6,11].

For the I.S., at the concentration level optimized for the method (25 ng mL^{-1}), a signal suppression of 10% was found for the selected dilution factor (n=10).

In general, the influence of the matrix effect is higher for the less retained analytes probably due to their coelution with polar compounds of urine.

3.3. Method validation

The method validation was carried out in terms of linearity, precision and accuracy, limits of detection (LOD) and quantification (LOQ). Calibration curves were prepared by spiking seven urine samples at five different concentrations in triplicate, in the range 0–100 ng mL^{-1} for all pteridines except XAN which was in the range 0–400 ng mL^{-1} due to its lower sensitivity (see Section 2.2.2.). After optimization of the MS parameters, MS response of the I.S. (6MPT) was firstly tested using 10–100 ng mL^{-1} solutions, as for the rest of the analytes. Considering the MS response, a final concentration of I.S. of 25 ng mL^{-1} was considered to provide an optimum I.S. response and pteridine/I.S. peak area ratios of the EICs were used for quantification purposes.

Good linearity ($R^2 \geq 0.99$) was observed for all pteridines in all samples. Results of the least squares regression analysis and detection and quantification limits for all urine samples are summarized in Table 2. The lowest limit of detection was found for PT (between 1.70 and 3.88 ng mL^{-1}), while the highest limit was for XAN (between 10.5 and 49.9 ng mL^{-1}) indicating a wide range of sensitivity between the different analytes in the studied urine samples. These limits are higher than those obtained by

HPLC with fluorescence detection [7,16,30]. Nevertheless, the omission of a previous oxidation step, plus the possibility of determining dihydropteridines and pteridines in the same run, makes MS an interesting alternative to fluorescence detection.

The precision study was made by using external and internal standard methodology. As expected, repeatability improved when the internal standard was employed. Results obtained are shown in Table 3. Intra-day ($n=6$) and inter-day ($n=5$) precision study was realized for a standard mixture of pteridines in presence of diluted urine (1:10) at a concentration level included in the calibration range. The relative standard deviations (% RSD) ranged from 1.9 to 9.2% in the intra-day injections and from 2.8 to 17% in the inter-day precision study, when I.S. was used.

Table 3
Precision of the HPLC-ESI-MS proposed method.

Compound	Repeatability			
	Intra-day ($n=6$)		Inter-day ($n=5$)	
	RSD[a] (%)	RSD[b] (%)	RSD[a] (%)	RSD[b] (%)
NEO	4.6	2.7	12	2.8
NH2	10.5	9.2	26	17
MON	3.4	3.3	12	5.4
XAN	6.7	5.0	13	5.3
BH2	6.4	4.8	20	11
BIO	3.3	2.3	11	4.7
PT	3.7	2.5	17	12
6HMPT	2.9	2.5	9.5	9.1
ISO	5.0	1.9	7.3	4.6
PT6C	4.1	2.7	12	9.5
6MPT (I.S.)	2.8	–	11	–

[a] Calculated using the pteridine peak areas of the EICs.
[b] Calculated using the pteridine/I.S. peak area ratios of the EICs.

The worst RSD values were those obtained for hydropteridines, NH2 and BH2, in the inter-day assay. This behavior is probably due to the aerial oxidation of the reduced forms. A significant improvement in inter-day assay RSD values is observed for NEO when I.S. is used. These results demonstrate the precision of the developed method and the potential of the proposed approach for quantitative purposes.

3.4. Application to urine samples

The proposed method was applied to the analysis of seven urine samples. In this study CREA has been included because urinary CREA has to be used to normalize the ratio of excretion of the studied biomarkers. The CREA of urine samples was analyzed spectrophotometrically by applying the modified Jaffe method (see Section 2.6.). Pteridines/CREA and NEO/BIO ratio values were calculated employing the average value ($n=3$) obtained for pteridines and CREA in each urine sample. Results obtained are shown in Table 4. In all un-oxidized adult urines, the quantifiable pteridines were NH2, NEO and BH2. BIO was quantified in all samples except one, while PT was detected in all samples except two. ISO was quantified only in two urine samples corresponding to the youngest volunteers. XAN, MON, 6HMPT and PT6C were not detected in the optimized conditions. In all cases, dihydropteridinic forms were found in higher concentration than their oxidized forms.

Values concerning the content of pteridines in urine can be found in literature, primarily for NEO, BIO and NEO/BIO ratio, for healthy people and for different pathological cases. Data presented in this manuscript have been obtained from urine samples without prior pretreatment. Hence, the analysis of the hidropteridines and the oxidized pteridines separately is possible and the values of NEO and BIO given correspond to the naturally excreted

Table 4
Marker pteridines/creatinine and neopterin/biopterin ratios calculated in healthy human urine samples by the proposed HPLC-ESI-MS method.

	Ratio values[a]									
	NEO/CREA	**NH2/CREA**	**NEO_T/CREA**	**BH2/CREA**	**BIO/CREA**	**BIO_T/CREA**	**PT/CREA**	**ISO/CREA**	**NEO/BIO_T**	**NEO_T/BIO_T**
Woman (51)[b]	0.111	0.576	0.687	0.424	0.058	0.482	0.106	< LOD	0.230	1.425
Man (51)[b]	0.074	0.389	0.462	0.118	0.065	0.183	< LOD	< LOD	0.404	2.525
Man (50)[b]	0.161	0. 939	1.100	0.381	< LOQ	—	0.124	< LOD	—	—
Woman (33)[b]	0.215	1.426	1.641	1.286	0.174	1.460	0.121	< LOD	0.147	1.124
Woman (32)[b]	0.123	0.550	0.673	0.663	0.086	0.749	< LOD	< LOD	0.164	0.899
Woman (22)[b]	0.232	1.164	1.396	0.625	0.107	0.732	0.082	0.089	0.317	1.907
Woman (17)[b]	0.117	0.490	0.607	0.860	0.084	0.944	0.055	0.052	0.124	0.643

Note: NEO_T = NEO+NH2; BIO_T = BIO+BH2.
[a] Calculated with the average value ($n=3$) obtained for pteridine and creatinine in each urine sample and expressed as mmol pteridine/mol CREA.
[b] Age.

Table 5
Values of NEO/CREA and NEO/BIO ratios in urine samples (mmol pteridine/mol CREA) reported in literature.

	NEO/CREA	**NEO_T/CREA**	**NEO/BIO_T**	**NEO_T/BIO_T**	**Treatment of urine**	**Ref.**
Pathological children urines (< 10 years old)	0.29–1.08	—	0.32–0.83	—	Unoxidized urine	[7]
	0.06–1.05	—	—	—		[31]
Pathological adult urines (> 25 years old)	—	0.21	—	2.59	Pre-oxidation with I_2/I^-	[6]
Healthy children urines (< 10 years old)	0.21	—	0.12	—	Un-oxidized urine	[7]
	—	0.39	—	0.86	Pre-oxidation with I_2/I^-	[16]
	—	0.46	—	0.57	Pre-oxidation with $KMnO_4$	
Healthy adult urines (> 25 years old)	0.094	—	0.12	—	Un-oxidized urine	[7]
	—	0.20	—	0.98	Pre-oxidation with I_2/I^-	[16]
	—	0.21	—	0.45	Pre-oxidation with $KMnO_4$	
	—	0.12	—	1.27	Pre-oxidation with I_2/I^-	[6]
Healthy adult urines (<25 years old)	0.17	1.00	0.22	1.28	Un-oxidized urine	*Proposed method*
Healthy adult urines (>25 years old)	0.14	0.91	0.24	1.49		

compounds in their oxidized forms. However, urine can be previously oxidized and then, the total pteridines content (hydropteridines plus pteridines) is determined and the values of NEO_T (NH2 + NEO) and BIO_T (BH2 + BIO) are reported. These events generate some confusion when consulting data concerning the content of pteridines in biological fluids.

Pteridine/CREA ratios (expressed as mmol pteridine/mol CREA) in underivatized and derivatized urine, reported in literature are summarized in Table 5. In the direct analysis of urine samples, the values of NEO/CREA ratios were 0.094 and 0.21 for healthy adults and children respectively [7]; 0.29–1.08 [7] and 0.063–1.05 [31] for pathological children; NEO/BIO_T ratio values of 0.12 for healthy adults and children and 0.32–0.83 for pathological children were also reported [7].

Different reagents have been investigated for the oxidation of urine samples and the reported pteridine levels vary slightly. Thus, NEO_T/CREA ratios of 0.12 and 0.21 and, NEO_T/BIO_T ratios of 1.27 and 2.59 for healthy volunteers and cancer patients, respectively, were reported using CE-LIF, previous oxidation with I_2/I^- [6].

NEO_T/CREA ratios of 0.20 and 0.39 for healthy adults and children for preoxidation using iodine/iodine, and 0.21 and 0.46 for healthy adults and children using permanganate were described using HPLC [16]. NEO_T/BIO_T ratios of 0.98 and 0.86 for healthy adults and children were obtained when iodine/iodine pre-oxidation was employed and 0.45 and 0.57 for healthy adults and children when permanganate was used [16].

Results summarized in Table 5 show mean values of the proposed method of 0.14 and 0.17 for NEO/CREA ratio; 0.91 and 1.0 for NEO_T/CREA ratio; 0.24 and 0.22 for NEO/BIO_T ratio and 1.49 and 1.28 for NEO_T/BIO_T ratio, for healthy adults younger and older than 25 years old, respectively. These values are comparable to those reported in literature taking into account the aged and sex diversity.

4. Conclusions

The determination of pteridines (disease markers) has become highly important to establish their variation in pathological states. The proposed method allows the simultaneous determination of marker pteridines in urine samples through a chromatographic system with MS detection. MS instrumental parameters were optimized and with the aim of minimizing sample handling, standard addition calibration method was applied to diluted urine samples without a previous sample pretreatment step. The results obtained in the present work indicate that dihydropteridines are the predominant forms in untreated urine of healthy adults. NEO/CREA ratios, in un-oxidized urines of healthy volunteers, are similar to those found in literature and lower than values reported in pathological urine samples. Although NEO_T/CREA ratio calculated by the proposed method is higher than those found by pre-oxidation of urines, NEO_T/BIO_T ratios are similar to bibliographic data. The main advantages of this method are its simplicity, the use of cheap disposable reagents and materials and the small volume of organic solvent consumed, being the protocol straightforward and environmentally friendly.

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