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Short communication

Quantitative measurement of plasma free metanephrines by ion-pairing solid phase extraction and liquid chromatography–tandem mass spectrometry with porous graphitic carbon column

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ABSTRACT

Plasma free metanephrine and normetanephrine are the best biomarkers for diagnosing pheochromocytoma. In the past few years, liquid chromatography–tandem mass spectrometry has become the preferred technology to measure plasma metanephrine and normetanephrine because of its high sensitivity and specificity, as well as fast and simple sample preparation. In this study, we report a liquid chromatography–tandem mass spectrometry method for measuring plasma metanephrine and normetanephrine. A solid phase extraction method using ion-pairing reagent and C18 stationary phase was used for sample preparation. We tested a porous graphitic carbon column and a HILIC column for chromatographic separation, and the former one showed better resolution with no interference from plasma matrix. This method was linear from 7.2–486.8 pg/mL for metanephrine and 18.0–989.1 pg/mL for normetanephrine with an accuracy of 92.2–111.8% and 92.1–115.0%, respectively. Inter-assay and intra-assay CV for metanephrine and normetanephrine at two different concentration levels ranged from 2.0% to 10.9%. In conclusion, this liquid chromatography–tandem mass spectrometry method using ion-pairing solid phase extraction and porous graphitic column was simple and efficient for measuring plasma metanephrines.

1. Introduction

Pheochromocytoma are rare tumors that secrete large amount of catecholamines in the adrenal medulla [1]. Catecholamines are metabolized to metanephrines which include metanephrine (MN) and normetanephrine (NMN) [1]. Compared to plasma catecholamines and urine catecholamines/metanephrines, plasma free metanephrines (unconjugated forms of metanephrine and normetanephrine in plasma, referred as Pmets in the following text) were shown to have the highest sensitivity and specificity in diagnosing pheochromocytoma using standard procedures as gold standards including imaging techniques and/or pathological examination of surgically removed tumor specimens [2,3]. Therefore, Pmets measurement is considered the best biochemical test for diagnosing pheochromocytoma [2]. Historically, Pmets have been measured by high performance liquid chromatography coupled with electrochemical detection (HPLC-ECD), colorimetric/enzymatic immunoassays and gas chromatography mass spectrometry (GC–MS) [4–9]. HPLC-ECD method requires laborious sample preparation and extensive chromatography time, and is prone to interferences [4]. Inconsistency has been observed between different enzymatic immunoassays [7]. GC–MS often lacked sufficient sensitivity due to the low concentrations of circulating Pmets [9].

Liquid chromatography tandem mass spectrometry (LC–MS/MS) has been increasingly used to measure Pmets in the past few years because of its high selectivity, sensitivity and relative simple sample preparation [8,10–15]. While there has been one report using isopropanol protein precipitation before LC–MS/MS analysis [14], the most commonly used sample preparation method is solid phase extraction (SPE). Pmets are very polar; therefore, special SPE and chromatographic methods have been developed for their analysis. Lagerstedt et al. used a mixed mode (hydrophilic–lipophilic balance, or HLB) SPE for sample preparation and a cyano LC column for chromatographic separation [15]. Peaston et al. used weak cation exchange (WCX) SPE cartridges for sample clean-up, and the subsequent LC–MS/MS analysis used hydrophilic interaction chromatography (HILIC) [12]. A similar strategy was used by de Jong et al. with the modification that the WCX SPE extraction was performed on-line [11]. Another

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special SPE method was reported by Talwar et al. for the purification of urine catecholamines, which are structurally similar to metanephrines [16]. In that method, diphenyl boronate was used to form a complex with catecholamines in alkaline condition, and the complex could be retained by the reverse phase (RP) SPE cartridges even under organic washing conditions. The bound catecholamines were released under acidic conditions which disrupted the complex.

Ion-paring (IP)-SPE has been used to purify a wide range of polar compounds including sugars, amino acids, phenols, biogenic amines, nucleosides/nucleotides, and triacylglycerides [17,18]. Ion-paring reagents have both polar and non-polar moieties. The polar portion can form non-covalent interaction with polar compounds of interest. The resulting complex can then be readily retained by RP SPE cartridges due to the non-polar portion of the ion-pairing reagent [19–22]. To the best of our knowledge, IP-SPE has not been tested for Pmets purification. Because Pmets have an amine group, volatile halogenated medium-alkyl chain carboxylic acids (e.g. trifluoroacetic, perfluorobutyric, and perfluoroheptanoic acids) can be used to form pairs with Pmets.

Porous graphitic carbon (PGC) columns are made of 100% carbon, and are chemically very stable and therefore can be routinely used at very high temperatures (up to 200 °C) to obtain better chromatographic peaks. The retention mechanism involves a charge-induced interaction of the polar analyte with the polarizable surface of the graphite. The PGC column is ideal for retaining and resolving very polar and hydrophilic molecules, which are normally not retained very well on reversed-phase columns using typical MS compatible mobile phases. Commercial PGC columns have been used to separate a variety of polar compounds [23–27] including catecholamines [27]. In this study, we aimed to develop an LC–MS/MS method for measuring Pmets using IP-SPE and PGC column chromatography. For comparison purposes, we have also evaluated a HILIC column.

2. Experimental

2.1. Materials and reagents

HPLC grade acetonitrile, water, isopropanol, formic acid, methyl *tert*-butyl ether (MTBE) and acetone were purchased from Fisher Scientific (Houston, TX, USA). Heptafluorobutyric acid (HFBA, 99%), perfluoroheptanoic acid (PFHA, 99%), ammonium formate (NH_4FA, >99.995%), epinephrine, metanephrine, and normetanephrine were from Sigma–Aldrich (St. Louis, MO, USA). The isotopically labeled internal standards, D,L-metanephrine-d3·HCl (deuterated N-methyl group; 98%D) and D,L-normetanephrine-d3·HCl (deuterated N-methyl group; 97%D) were from Medical Isotopes (Pelham, NH, USA). Stock solution of each compound was prepared in 0.1 N HCl and stored at −80 °C. HyperSep C18 (100 mg/1 mL) SPE cartridges, Hypercarb PGC column (50 mm × 2.1 mm, 5 μm particle size), Hypersil Gold HILIC column (150 mm × 3 mm, 5 μm particle size) were from Thermo Hypersil-Keystone (Bellafonte, PA, USA). Human charcoal stripped serum was purchased from Equitech Bio (Kerrville, TX, USA). Pooled and individual human plasma collected in potassium ethylenediaminetetraacetate (K_2EDTA)-coated tubes were purchased from Bioreclamation (Hicksville, NY, USA).

2.2. Calibrators and internal standards

Calibrators were prepared by spiking charcoal stripped serum with metanephrine stock solutions. Endogenous metanephrines were not detected in the charcoal stripped serum (data not shown). Five levels of calibrators were prepared with concentrations of 7.81, 15.6, 31.3, 125, and 500 pg/mL for MN, and 15.6, 31.3, 62.5, 250, and 1000 pg/mL for NMN. All calibrators were aliquoted and stored at −80 °C. An internal standard (IS) working solution was prepared in 0.1 N HCl, in which, MN-d3 and NMN-d3 concentrations were 5.0 and 20.0 ng/mL respectively. The final concentrations of MN-d3 and NMN-d3 in samples are 250 and 1000 pg/mL, respectively.

2.3. Ion-pairing solid phase extraction

SPE was performed using a vacuum manifold (Fisher Scientific). Briefly, 40 μL of IS working solution was added to 0.5 mL of plasma sample. HyperSep cartridges were preconditioned with acetonitrile (1 mL × 1) and 0.1% PFHA in water (v/v, 1 mL × 2) before samples were loaded. Cartridges were washed with 0.1% PFHA in water (v/v, 1 mL × 2) and eluted with 60% acetonitrile in water (v/v, 0.2 mL × 3). Pressure inside the vacuum manifold was kept at 22 mmHg during all SPE steps. The eluate was dried under N_2 stream at 37 °C for 50 min, and reconstituted with 0.2 mL 2% formic acid in water. The mixture was centrifuged at 15,000 × *g* for 10 min, and 40 μL of supernatant was injected for LC–MS/MS analysis.

2.4. Liquid chromatography and tandem mass spectrometry

The LC–MS/MS system was composed of a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with an Accela 1250 quarternary LC pump (Thermo Scientific) and a CTC Pal autosampler. Software included Xcalibur 2.1 and LCQuan 2.6 (Thermo Scientific, San Jose, CA, USA).

For the PGC column chromatography, column temperature was maintained at 70 °C, and three mobile phase (MP) buffers (MP1, MP2 and MP3) were used. MP1 was 50 mM NH_4FA and 1% formic acid in water. MP2 was 0.1% formic acid in acetonitrile. MP3 was 9:9:2 (v/v/v) mix of acetonitrile/isopropanol/acetone (v/v/v). After injection, the column was washed with 98% MP1/2% MP2 for 0.5 min at flow rate of 1 mL/min, and then the percentage of MP2 increased to 40% using a linear gradient within 5 min at a flow rate of 0.5 mL/min. After the gradient, the column was washed with 100% MP2, 100% MP3 and 98% MP1/2% MP2 for 1 min, 0.5 min and 3 min respectively, at flow rate of 1 mL/min. Data acquisition started 2 min and ended at 4 min. The mass spectrometer was operated in the positive electrospray ionization mode. Optimization of ion source parameters was performed by infusing a metanephrine solution (1 μg/mL in 1:1 mix of methanol/acetonitrile) at a constant flow rate (5 μL/min) into a LC stream (0.5 mL/min) of 75% MP1/25% MP2. The resulting parameters were as follows: a spray voltage of 1500 volts, a vaporizer temperature of 480 °C, a sheath gas pressure of 30 a.u., an auxiliary gas of 5 a.u., and a capillary temperature of 380 °C.

For the HILIC chromatography, two mobile phase buffers (MPA and MPB) were used. MPA was 95:5 mix of acetonitrile/100 mM NH_4FA (pH 3.2), and MPB was 50:45:5 mix of acetonitrile/water/100 mM NH_4FA (pH 3.2). After sample injection, the column was maintained at 100% MPA for 2.5 min at a flow rate of 1 mL/min, and the content of MPB was step increased to 20% and maintained at 20% for 3.5 min at a flow rate of 1 mL/min. The column was then washed with 100% MPB for 1.5 min at a flow rate of 2 mL/min and then equilibrated with 100% MPA for 7.5 min at flow rate of 1 mL/min. Data acquisition was between 2.5 and 6.0 min. The mass spectrometer was operated in the positive electrospray ionization mode. The MS parameters were composed of a spray voltage of 4000 V, a vaporizer temperature of 480 °C, a sheath gas pressure of 30 a.u., an auxiliary gas of 5 a.u., and a capillary temperature of 380 °C.

For both methods, collision gas pressure was set at 1.5 mTorr for all analytes. SRMs, S-Lens value (V) and collision energy (V) were set at 180.1 → 147.8 *m/z*, 61 and 16 for MN, 183.1 → 150.8 *m/z*, 62 and 16 for MN-d3, 166.1 → 134.0 *m/z*, 52 and 15 for NMN, and 169.1 → 137.0 *m/z*, 56 and 15 for NMN-d3. Scan time of each SRM transition

was set at 100 ms. The Q1 and Q3 resolutions (FWHM) were set at 0.7 mass unit.

2.5. Method validation

2.5.1. Interference

Epinephrine was tested for interference because MS/MS could not differentiate it from NMN.

2.5.2. SPE recovery

SPE recovery was assessed with a post-extraction spike method. Briefly, charcoal stripped serum samples were spiked with MN, NMN, MN-d3 and NMN-d3 at 200, 400, 400, and 1600 pg/mL and subjected to IP-SPE in triplicates. Three additional aliquots of un-spiked charcoal stripped serum (0.5 mL) were processed with IP-SPE. To each eluate, equivalent amounts of MN, NMN, MN-d3 and NMN-d3 were added. After LC–MS/MS analysis of all samples, absolute recovery was calculated based on absolute peak areas while the relative recovery was based on the analyte/IS ratios.

2.5.3. Ion suppression

To assess ion suppression, we performed a post-column infusion experiment [28] in which a constant flow (5 μL/min) of MN-d3 or NMN-d3 (both at 100 ng/mL) was infused post-column into the mobile phase using a T junction while IP-SPE extracted human plasma (without internal standards) or mobile phase buffer (blank) were injected. SRM of the injected IS was monitored for the entire LC gradient. The obtained chromatograms were visually inspected for obvious ion suppression or enhancement.

2.5.4. Mixing study

In order to evaluate whether charcoal stripped serum could be used as the diluent in the following linearity experiment, a mixing study was performed. Three sets of samples were prepared using IP-SPE. The first set had 6 individual human plasma samples, and the second set was 6 charcoal stripped serum samples spiked with 100 pg/mL of MN and 200 pg/mL of NMN. The third set was 1:1 mix ($n = 6$) of the first two sets of samples. After LC–MS/MS analysis, measured analyte/IS peak area ratio of each set 3 sample was compared to its expected value which was the mean of the corresponding set 1 and set 2 samples. A difference $\leq$20% was considered acceptable.

2.5.5. Linearity

Charcoal striped serum was spiked with MN and NMN to achieve a final concentration of 500 pg/mL and 1000 pg/mL, respectively. A serial 2-fold dilution with charcoal stripped serum was performed to make 8 levels of linearity samples with concentration ranges of 500–3.9 pg/mL and 1000–7.8 pg/mL for MN and NMN, respectively. Linearity samples were analyzed in triplicates along with one set of calibrators. The calibration curve was constructed by plotting the analyte:IS peak area ratio vs. analyte concentration. The linearity, accuracy and CV were calculated using Excel (Microsoft, Redmond, WA, USA). The lower limit of quantification (LLOQ) and the higher limit of quantification (HLOQ) were determined as the lowest and the highest concentrations in the linear range that gave accuracy within 100 ± 20% and a CV less than 20%.

2.5.6. Carryover

Charcoal stripped serum was spiked with MN and NMN to create a high level sample (500 and 1000 pg/mL for MN and NMN, respectively) and a low level sample (7.8 and 15.6 pg/mL for MN and NMN, respectively). After sample preparation, the low level sample was injected first (Low1) for LC–MS/MS analysis followed by the injection of the high level sample (High). Immediately after, the low level sample was re-injected (Low2). No significant carryover was determined when the difference between Low1 and Low2 was <20%.

2.5.7. Precision

Both human plasma samples and spiked charcoal stripped serum were used to evaluate precision. Charcoal stripped serum was spiked with MN and NMN at two levels (25 and 250 pg/mL for MN, 50 and 500 pg/mL for NMN, respectively). These samples were aliquoted and stored at −80 °C until use. Intra-assay CV was determined by running these two levels of samples five times within a batch. Inter-assay CV was determined by running the same two levels of samples five times a day for three days. Pooled human plasma was only tested for intra-assay CV ($n = 5$).

3. Results and discussion

3.1. Optimization of sample preparation

Among the two volatile ion-pairing reagents (HFBA and PFHA) we tested, PFHA gave better recovery of Pmets (data not shown). Therefore, it was used in the subsequent experiments. To minimize elution of interfering lipophilic compounds, several SPE elution buffers (20%, 40%, 60% and 80% acetonitrile in water) were tested and recovery of metanephrines reached maximum at 60% acetonitrile.

Effective removal of interfering substances from biological matrix is a key to a successful LC–MS/MS measurement of Pmets. During IP-SPE, plasma samples were loaded onto C-18 cartridges preconditioned with 0.1% PFHA. Although metanephrine and normetanephrine are highly polar and hydrophilic, their free amine groups could interact with the acidic groups of PFHA and the resulting Pmets-PFHA complex could be retained by C18 stationary phase. Presumably, polar compounds unable to form ionic complex with PFHA would be removed with aqueous washes.

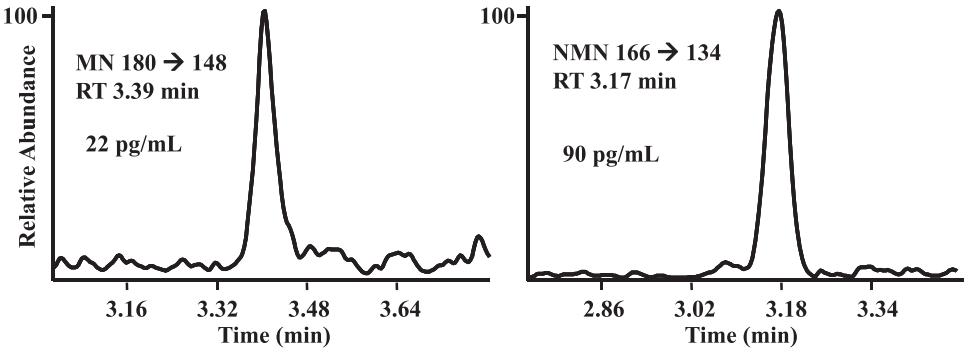

Fig. 1. Representative SRM chromatograms of MN and NMN using a processed human plasma sample. The PGC column was used. Retention time (RT), SRM transitions, and the measured concentrations are shown.

Table 1
SPE recovery.

In charcoal stripped serum	Spike before SPE[a] (mean ± CV)	Spike after SPE[b] (mean ± CV)	Absolute recovery (%)	Relative recovery (%)
MN (n = 3)	22,865 ± 13.9%	25,265 ± 9.3%	90.5	97.7
NMN (n = 3)	11,165 ± 11.1%	11,453 ± 12.5%	97.5	113.5
MN-d3 (n = 3)	27,809 ± 7.2%	30,140 ± 12.9%	92.3	n.a.
NMN-d3 (n = 3)	22,627 ± 9.2%	26,192 ± 4.5%	86.4	n.a.

[a] Measured peak area of charcoal stripped serum spiked with 100, 400, 400, and 1600 pg/mL of MN, NMN, MN-d3 and NMN-d3 before SPE.
[b] Measured peak area when equivalent amounts of above compounds were spiked after SPE.

SPE eluate was dried and reconstituted in an aqueous buffer (MP1). Usually, particular substances (presumably lipophilic compounds insoluble in aqueous buffer) were observed, especially more in processed human plasma samples. Therefore, samples were centrifuged, and the clear supernatant was injected for LC–MS/MS analysis.

3.2. Column chromatography

Both MN and NMN were retained and separated on the PGC column (Fig. 1). In order to obtain a symmetric and reproducible peak, a high column temperature (70 °C) was needed. No adverse effect of this high temperature on column performance was observed after >200 injections. In addition, analysis of an aqueous solution containing 1 ng/mL metanephrines before and after spiking 1 ng/mL catecholamines showed no conversion of catecholamines to metanephrines under this acidic condition with elevated temperature demonstrating that there was no significant methylation reaction of catecholamines' benzylic hydroxyl groups with methanol in the mobile phase (data not shown). It is also noteworthy that the retention times of MN and NMN were ~0.3 min earlier when samples prepared in neat solutions were injected directly. We suspect that this was probably due to the residual PFHA in IP-SPE processed samples which provided extra retention for both MN and NMN. This extra retention was beneficial due to better chromatographic separation and ionization.

In addition to the PGC column, a HILIC column was also tested because of a previous claim of better ionization efficiency [11]. Initially, dried SPE eluate was reconstituted with methanol before LC–MS/MS injection, and significant ion suppression was observed with processed human plasma samples (data not shown). This ion suppression was probably due to lipophilic substances not removed by IP-SPE. Therefore, we decided to dissolve the dried SPE eluate in water with 2% formic acid, and then performed liquid–liquid extraction with MTBE. Such treatments greatly cleaned up the samples and reduced ion suppression (data not shown). However, sample preparation was much longer, and peak shapes under the HILIC condition were not as symmetrical and reproducible (data not shown). Therefore, the following validation work was based on the method using IP-SPE and PGC column chromatography.

3.3. Method validation

3.3.1. Interference

Epinephrine and normetanephrine share the same SRM transitions and could not be differentiated just by MS/MS analysis. With PGC column chromatography, the epinephrine peak was baseline resolved (0.3 min apart) from the normetanephrine peak (data not shown).

3.3.2. SPE recovery

Extraction efficiency was assessed in charcoal stripped serum (n = 3). Absolute recovery of Pmets and IS ranged from 86.4% to 97.5%, and the relative recovery of MN and NMN was 97.7% and 113.5%, respectively (Table 1).

3.3.3. Ion suppression

Results from the post-column infusion experiments are shown in Fig. 2. Compared to injections of blanks, no obvious ion suppression was detected in the SRM chromatogram of MN-d3 using processed human plasma samples. However, apparent ionization enhancement of NMN-d3 was detected around its retention time.

3.3.4. Mixing study

The measured analyte/IS peak area ratio of 1:1 mixed samples were in line with expected values and the variations were from −16.5 to 10.6% for MN, and −15.6–1.4% for NMN. This indicated that metanephrine and normetanephrine originated from spiked charcoal stripped serum and those from human plasma behaved similarly relative to their corresponding IS during the whole process of IP-SPE and LC–MS/MS. Therefore, charcoal stripped serum was chosen as the diluent in the following linearity study, and as the matrix in the subsequent carryover and precision studies.

3.3.5. Linearity

The linearity range was determined to be 7.2–486.8 pg/mL for MN and 18.0–989.1 pg/mL for NMN. Within the linear range, accuracy ranged from 92.2% to 118.0% for MN, and from 92.1% to 115.0%

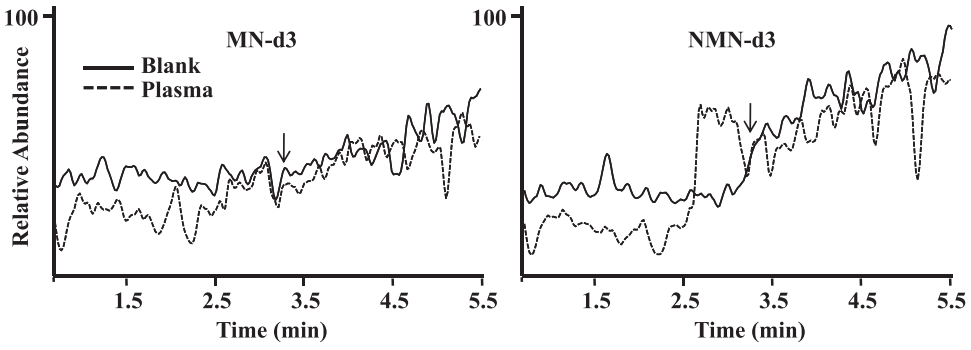

Fig. 2. Representative SRM chromatograms of post-column infusion of 100 ng/mL MN-d3 (top) and NMN-d3 (bottom) after injections of buffer blanks (solid lines) and processed patient pool (dash lines). No internal standards were added to patient samples. Arrows indicated retention times of MN and NMN.

Table 2
Precision data.

	MN		NMN	
	25 pg/mL	250 pg/mL	50 pg/mL	500 pg/mL
Intra-assay precision (%) $n = 5$	10.9	4.6	9.6	2.1
Accuracy (%)	98.9	96.9	110.2	90.9
Inter-assay precision (%) $n = 15$	10.3	6.5	10.6	5.6
Accuracy (%)	100.6	102.7	108.7	97.4

for NMN. CV ranged from 0.9% to 18.0% for MN, and from 2.1% to 13.2% for NMN. The determined LLOQ was 7.2 pg/mL for MN and 18.0 pg/mL for NMN.

3.3.6. Carryover

No significant carryover was observed by testing the spiked charcoal stripped serum samples with Low1 (6.6 pg/mL)-High (540.1 pg/mL)-Low2 (7.5 pg/mL) for MN and Low1 (16.0 pg/mL)-High (1016.4 pg/mL)-Low2 (16.1 pg/mL) for NMN.

3.3.7. Precision

Precision was first assessed with spiked charcoal stripped serum. Inter- and intra-assay CV values at two levels of both analytes varied between 2.1% and 10.9% (Table 2). Precision was also assessed with a human plasma pool (35.6 pg/mL of MN and 53.1 pg/mL of NMN, $n = 5$) and the determined intra-assay CV was 6.3% and 7.8% for MN and NMN, respectively.

4. Conclusions

A sensitive LC–MS/MS method was developed to quantify plasma metanephrines. Ion-pairing SPE was used for sample preparation, and the method was optimized to reduce both hydrophilic and lipophilic interfering substances. PGC column was used for chromatographic separation of metanephrines and it showed superior performance for these very polar compounds.

Disclosure statement

Xiang He, Yang Shi, and Marta Kozak are currently employed at ThermoFisher Scientific.

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