Metabolomic analysis of glycerophospholipid signatures of inflammation treated with non-steroidal anti-inflammatory drugs-induced-RAW264.7 cells using ^1H -NMR and U-HPLC/Q-TOF-MS

Xia Wu, Han Cao, Lifang Zhao, Jianao Song, Yuqi She, Yifan Feng*

Central Laboratory, Guangdong Pharmaceutical University, Guangzhou 510006, P. R. China

* Correspondence authors. Tel.: +86 20 39352522; fax: +86 20 39352523.
E-mail addresses: yffeng@139.com (Y. Feng)

ABSTRACT

Non-destructive proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy and highly sensitive ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (U-HPLC/Q-TOF-MS) coupled to data processing methods were applied to analyze the metabolic profiling changes of glycerophospholipids (GPLs) in RAW264.7 cells from inflammation to prognosis. Analysis of $^1\text{H-NMR}$ was shown that the models were grouped successfully, illustrating that all of them had significant differences. Based on the highly simple, accurate, non-targeted and non-destructively advantages of $^1\text{H-NMR}$, it could be used as a new screening tool of anti-inflammatory drugs in the metabolic profiling of GPLs. 58 GPLs were identified by U-HPLC/Q-TOF-MS, and 19 components were firstly identified in this study compared with our previous results. In addition, ten potential biomarkers were proved, of which phosphatidylcholine (PC) (16:0/18:1) and (18:0/18:1) changed consistently in three drug-induced groups and might be the important biomarkers. Compared with $^1\text{H-NMR}$, U-HPLC/Q-TOF-MS showed higher sensitivity and specificity and was more suitable for the determination of biomarkers apart from the deficiency of time-consuming sample preparation steps and unambiguous metabolite identification. Therefore, it is feasible to analyze the changes of GPLs during inflammation by combining $^1\text{H-NMR}$ spectroscopy with U-HPLC/Q-TOF-MS. The metabolic profiling of GPLs provides valuable evidence for inflammation diagnosis and prognosis, and might unravel the mechanisms involved in inflammation progression.

Keywords:

^1H -NMR

U-HPLC/Q-TOF-MS

Glycerolphospholipids

RAW264.7 cells

Chemometrics

Biomarkers

1. Introduction

Inflammation, as suggested by several epidemiological and clinical studies, plays a vital role in various high incidence diseases such as pulmonary vessels inflammation [1], diabetes mellitus type 2 [2], bronchial asthma [3], tumor [4] and atherosclerosis [5]. Non-steroidal anti-inflammatory drugs (NSAIDs), which exhibit potent anti-inflammatory, antipyretic and analgesic functions, have been widely applied to treat inflammation through inhibition of cyclooxygenase [6-7]. But they may also lead to numerous adverse reactions, notably those of the gastrointestinal tract [8]. In order to solve this problem, we tried to research glycerophospholipids (GPLs), one of the precursors of arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid, AA) [9]. As a fatty acid metabolized from GPLs [10], AA is generally esterified in inflammatory cells in the sn-2 position of GPLs, particularly phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) [11]. Normally, free AA is kept at very low levels in resting unstimulated cells and exceeded under the stimulation situation that of reincorporation into phospholipids in the activation of intracellular PLA2s and the accumulation of AA converted into eicosanoids [12-13]. Since GPLs compose 60% of membrane lipids, it is critical to investigate the relationship between GPLs and inflammation under NSAIDs intervention.

Nuclear magnetic resonance (NMR) spectroscopy and ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (U-HPLC/Q-TOF-MS) are the two main analytical techniques highly suitable for metabolomics applications which exhibit different analytical strengths and weaknesses [14-15]. Based on the advantages of non-targeted, non-destructive, reproducible and unbiased, NMR spectroscopy has been applied in metabolomics for disease diagnosis and drug development with a spectral resolution comparable to that of solution NMR spectroscopy [16-17]. Multivariate statistical analyses on NMR spectral data of biological samples have been generally applied to map the variations in metabolic profiles and generate statistical models for improving adjunct diagnosis or prognosis [18]. However, it is difficult for NMR to identify the components of complex lipids which contain constituents present in a wide concentration range with varying polarities. On the

other hand, because of its high sensitivity and resolution, U-HPLC/Q-TOF-MS has already become an indispensable technology for metabolomic analysis [19]. One of the advantages with U-HPLC/Q-TOF-MS compared with NMR is that many more metabolites with lower concentrations could be sensitively determined and quantified in complex samples. Nevertheless, this method has poor reproducibility and destructive nature. Therefore, the combined use of NMR and U-HPLC/Q-TOF-MS for metabolic profiling could provide more information on biological changes in an organism [20].

In this study, ^1H -NMR and U-HPLC/Q-TOF-MS were simultaneously employed to analyze the changes of glycerophospholipids (GPLs) in RAW264.7 cells. Three new types of NSAIDs (meloxicam, diclofenac sodium and nimesulide) were used to interfere with the inflammatory model of macrophages. In combination with the results of previous studies, we tried to screen the common change biomarkers. A rapid and non-destructive screening platform was firstly established for the metabolic profiling of GPLs by combining ^1H -NMR spectroscopy with PLS-DA and OPLS-DA. 10 potential biomarkers were found by U-HPLC/Q-TOF-MS combined with multivariate data analysis, of which PC (16:0/18:1) and PC (18:0/18:1) changed consistently in three drug-induced groups. The combined analytical methods provided much more information for the change of GPLs on treatment or disease state.

2. Materials and methods

2.1. Reagents

Mouse RAW264.7 macrophages were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS) and Dulbecco's phosphate-buffered saline (DPBS) were produced by Gibco (LA, USA). Penicillin-streptomycin solution was from Beijing Kangbei Yuan Technology Co., Ltd. (Beijing, China). All references (meloxicam, diclofenac sodium, nimesulide) were obtained from Guangdong Institute for Food and Drug Control (Guangzhou, China). KLA was purchased from Avanti (Alabaster, AL, USA). Tumor necrosis factor-alpha (TNF- α) kit was bought from the biological quality control laboratory of Ruikang Bo. (Tianjin, China). Chloroform-*d* was obtained from Qingdao Dragon (Qingdao, China). Methanol, isopropanol and acetonitrile (HPLC grade) were from Merck (Darmstadt, Germany). Acetic acid (HPLC grade) was obtained from Aladdin (Shanghai, China). A reference compound leucine-enkephalin was purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Cell culture

Mouse RAW264.7 macrophages were cultured in DMEM supplemented with 10% FBS and 25 $\mu\text{g/mL}$ penicillin-streptomycin solutions in a humidified atmosphere containing 5% CO_2 at 37 °C, and the culture medium was changed every two days.

2.3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Optimal dose of drugs was determined by MTT (Sigma) assay [21]. Cell suspensions containing about 8×10^3 cells 200 μL medium/well were added to 96-well plates and cultured in the above conditions for 12 h. The concentration gradient of each drug was as follows: 10, 20, 40, 60, 80 and 120 $\mu\text{g/mL}$ meloxicam solutions; 10, 30, 50, 80, 120 and 160 $\mu\text{g/mL}$ diclofenac sodium solutions; 20, 40, 60, 80, 120 and 160 $\mu\text{g/mL}$ nimesulide solutions. Every concentration was designed for 6 parallel wells

and cultured for 24 h. Then, 20 μ L of MTT was added and incubated for 4 h at 37 °C, and 150 μ L of DMSO was added to dissolve the blue formazan crystals. After 10 min of slow shaking, they were measured by spectrophotometry at a wavelength of 490 nm with Microplate (Bio-Rad, Hercules, CA, USA).

2.4. Cell grouping

Cells were seeded into dishes at the density of 4×10^5 /mL and cultured approximately for 12 h. Then the cells were randomly divided into five groups: blank group (K), Kdo2-lipid A (KLA)-induced inflammation group (Y), and NSAIDs-induced groups (meloxicam group, M; diclofenac sodium group, S; nimesulide group, N). Experiment for each group was performed in five replicates. Groups M, S, N were cultured with meloxicam, diclofenac sodium and nimesulide at the optimal concentration respectively and groups K and Y were cultured in the medium for 1 h. Then group Y and NSAIDs-induced groups were incubated with KLA to the final concentration with 100 ng/mL, and equal-volume DPBS was added in group K. All of them were incubated for 24 h under the conditions mentioned above.

2.5. TNF- α analysis

The secretion of TNF- α was measured with an ELISA kit, and the model was validated according to the instructions. Standard curve was plotted with the data at 100, 250, 500, 1000 and 2000 pg/mL respectively. Groups K, Y, M, S and N were obtained at the indicated time and subjected to ELISA. Cytokine levels in the supernatant were analyzed in the sensitive range of standard curve.

2.6. Lipid extraction

After incubation, the cells were centrifuged at 600 g for 5 min, and the supernatant was then discarded. Lipid was extracted by using a modified method of Bligh and Dyer with chloroform-methanol-0.1 mol/L HCl (1:1:1, v/v/v) below 4 °C [22]. The chloroform phase was separated by being centrifuged at 16000 g and evaporated under nitrogen. The residue was re-dissolved by deuterated

chloroform (0.6 mL) and transferred to a 5 mm NMR tube for ^1H -NMR spectroscopic analysis. After ^1H -NMR determination, the samples were evaporated under nitrogen and re-dissolved by 50 μL of chloroform-methanol (9:1, v/v) for U-HPLC/Q-TOF-MS analysis. All the above operations were completed below 4 $^{\circ}\text{C}$.

2.7. ^1H -NMR spectroscopic analysis

A Bruker Avance III NMR spectrometer operating at 500 MHz for ^1H observation and a 5 mm BBFO broadband probe were used. All pulse sequences were got from Bruker pulse procedure library. ^1H -NMR spectra were acquired at 293.5 K (20.35 $^{\circ}\text{C}$) with a 16 ppm spectral width, a 1 s relaxation delay (d1), and 256 scans (2 dummy scans). The 90° pulse width was adjusted to 14.3 μs for each sample, and a total of 64 transients were acquired into 64 k data points. The acquired data were processed using TOPSPIN 2.0 software (Bruker AG, Fällanden, Switzerland). A 0.3 Hz line-broadening function was applied to all spectra prior to Fourier transformation and the frequency domain spectra were manually phased and baseline corrected, referenced to TMS at δ 0.

2.8. Data analysis for ^1H -NMR spectroscopy

NMR spectra were carefully aligned using MestReNova (Version 8.0, Mestrelab Research SL). The spectral region of each metabolite was integrated into one binning and normalization. Prior to statistical analysis, all spectra were automatically baseline-corrected and binned to 0.1 ppm spectral width. The spectral regions of low significance were excluded with MestReNova. Finally, specific normalization was applied for all the samples. The resulting data matrix was conducted using SIMCA-P version 12.0 software package with pareto-scaled prior to the supervised orthogonal projections (PLS-DA and OPLS-DA). Quality of the models was evaluated with the relevant R^2 and Q^2 discussed elsewhere [23]. All statistically significant differences in mean values were tested by one-way ANOVA for comparisons of multiple groups. The differences were considered significant when the value of p was lower than 0.05.

2.9. U-HPLC condition

ACQUITY U-HPLC/Q-TOF-MS system (Waters, USA) equipped with binary Pump, vacuum degasser and autosampler was controlled with MassLynx™ (V4.1) software (Waters, Manchester, UK). An ACQUITY UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm; Waters) by setting the column heater at 50 °C was applied for chromatographic separation. All samples were saved at temperature of 4 °C in the sample room. Mobile phases contain 10 mM ammonium acetate and 0.1% acetic acid (A) and 50% intermixture of acetonitrile and isopropanol (B), and use a gradient elution program of 39-18.6% A at 0-4 min, 18.6-10% A at 4-20 min, 10-0% A at 20-21 min, 0-0% A at 21-24 min, 0-39% A at 24-25 min, 39-39% A at 25-28 min at flow rate of 0.3 mL/min.

2.10. Q-TOF-MS parameters

ACQUITY U-HPLC/Q-TOF-MS system was configured with an ESI ion source operating in positive ion (ESI+) and negative ion (ESI-) modes. High-purity nitrogen (N₂) was applied as desolvation gas at a flow rate of 350 L/h and ultrahigh-purity helium (He) as collision gas. Flow rate of cone gas (N₂) was 50 L/h. De-solvation and source temperature was set at 350 and 120 °C, respectively. Both of capillary voltage and cone voltage were set to 3200 V for ESI (+/-) and sample cone was 30 V. Acquired data was corrected via an external reference (Lock-Spray™) leucine enkephalin with 556.2771 in ESI (+) and 554.2615 in ESI (-) to ensure accuracy during MS analysis. Spectra were acquired in the range of *m/z* 420-950 for full scan time MS analysis.

2.11. Data analysis for U-HPLC/Q-TOF-MS

U-HPLC/Q-TOF-MS data sets were extracted and aligned by MarkerLynx™ application manager (V 4.1) software system for further discrimination of potential variables. Peak areas of samples were set to 10000. The data with mass higher than 80% were selected because a variable had a nonzero measurement value in at least 80% of the variables within one of the two subsets, the variable was included in the data set; otherwise the variable was automatically deleted. This procedure will be

referred to as the “80% rule” [24]. Using this rule, missing values or zero values caused by peaks that were not present in the sample/chromatogram were reduced. In order to effectively reduce the interference of meaningless information, ultimate data time was ranged from 0.6 min to 21 min. SIMCA-P version 12.0 software package was employed to visually discriminate the five groups. In pattern of PLS-DA, a statistically significant threshold of variable influence on projection (VIP) values was combined for the differential components, and the value of VIP exceeding 1.0 was identified and collected for U-HPLC/Q-TOF-MS analysis [25]. All statistically significant differences in mean values were tested by one-way ANOVA for comparisons of multiple groups. The differences were considered significant when the value of p was lower than 0.05.

3. Results and discussion

3.1. MTT assay and KLA-induced TNF- α production in RAW264.7 cells

Optimal dose of each drug was determined by MTT assay [26]. The concentrations of 30 $\mu\text{g/mL}$ meloxicam solution, 50 $\mu\text{g/mL}$ diclofenac sodium solution and 45 $\mu\text{g/mL}$ nimesulide solution respectively were used thereafter. TNF- α is a pro-inflammatory cytokine produced from inflammatory cells. It has direct effects on vascular endothelial cells to induce chemotactic factors, other cytokines, cells adhesion molecules and to facilitate the infiltration of leukocytes, all of which contribute to the inflammatory process [27-28]. The physiological level of TNF- α was measured for confirming whether to establish a successful model. The experimental results were taken from the reports of our previous work [29].

3.2. PLS-DA and OPLS-DA analyses of ^1H -NMR-based metabolic profiling data

^1H -NMR metabolic spectrum of phospholipid components of normal RAW264.7 cells was shown in Fig. 1, and some functional groups were identified by chemical shifts. ^1H -NMR spectra of GPLs in five groups were obtained, as shown in Fig. 2. It is difficult to observe immediately the information of

metabolic changes from the figure. Thus, data were introduced to SIMCA-P software package (version 12.0; Umetrics, Umeå, Sweden) for multivariate statistical analysis.

The metabolic profiles of samples were analyzed by PCA method in our previous study [29]. PCA as an unsupervised analysis model could only make a preliminary classification to raw data, and in this study the data were further analyzed by use of supervised PLS-DA and OPLS-DA analyses for improving the classification of data. PLS-DA scores plot of blank group, diclofenac sodium group versus inflammation group is shown in Fig. 3A. Full binned spectral region showed that blank group and diclofenac sodium group were basically separated from inflammation group whereas blank group and diclofenac sodium group overlapped. We speculated that the drugs induced the cells to undergo close-to-normal biochemical metabolism. In addition, high R^2X , R^2Y and Q^2 (cum) values of 0.931, 0.958, and 0.777, respectively, also indicated a good model with suitability and predictability. Score plots of PLS-DA for blank group, nimesulide group versus inflammation group and blank group, meloxicam group versus the inflammation group from ^1H -NMR spectroscopy are exhibited in Fig. 3B and C. The three groups of samples were also grouped successfully. The results of OPLS-DA analysis are shown in Fig. 4. It could be observed that inflammation group was completely separated from blank group and diclofenac sodium group, which distributed in the left and right sides of the score chart and were more concentrated (Fig. 4A). The credibility and weight coefficient of variables could be observed from Fig. 4B. More distributed in the "S" at the two ends of the contribution rate of variables is also greater. Besides, the other groups (Fig. 4C and 4E) were also grouped successfully.

The results of multidimensional analysis indicated that phospholipids metabolism had yielded significant differences (95% confidence interval) due to treatment group and inflammation group no overlapping. Diclofenac sodium group and blank group have large area overlap, and the other treatment groups and blank group have a small area of overlap, indicating that phospholipids metabolism of treatment group had tends to normal levels. Overlap degree of different dosing cells and normal cells in phospholipids levels close is different. The greater the overlap rate has on behalf of the greater approaching degree. Diclofenac sodium group and blank group almost completely overlap, indicating

that phospholipids metabolism is very close. The differences of approach degree mainly come from the factors such as drug potency, half life and tmax. In short, combining ^1H -NMR spectroscopy with multidimensional analysis based on total GPLs profiling was able to get high-throughput and parallel data in discriminating different states of cells. Analyzing total GPLs with ^1H -NMR spectroscopy allows rapid initial screening of anti-inflammatory drugs, depending on the differences between chemical groups. This rapid, simple, cheap and non-destructive method is ideal for screening large quantity of drugs.

3.3. U-HPLC/Q-TOF-MS analysis of GPLs components in the membrane

All of these groups were operated on positive and negative full scan modes. Total ion chromatogram (TIC) of each group is shown in Fig. 5. The chromatograms achieved a great separation between different classes of GPLs. Based on retention time and MS/MS fragmentation information of phospholipids molecules, 58 GPLs were identified, including 21 PCs, 15 LPCs, 2 PEs, 15 LPEs, 3 PGs, 1 PS, 1 PA (Table 1). 19 components were firstly identified in this study compared with our previous results [30].

3.4. Discrimination among GPLs components in five groups by U-HPLC-based metabolic profiling

Despite the obvious advantages, it was difficult for ^1H -NMR to directly observe the intensity changes of each GPL component due to resonance overlap. Thus, U-HPLC/Q-TOF-MS was applied combined with chemometrics. PCA was performed on normalized TIC peak areas to discriminate samples and view the holistic distribution and clustering. By utilizing the score plots with first two principal components (Fig. 6), samples were basically separated. The $R^2\text{X}$ and $R^2\text{Y}$ (cum) values > 0.6 and Q^2 (cum) > 0.5 indicated a good model (Fig. 6). Obviously, two methods (^1H -NMR and U-HPLC/Q-TOF-MS) enabled consistent outcomes. In the other hand proved that ^1H -NMR was suitable for GPLs as a rapid and non-destructive identification method. As a mature supervised clustering method, PLS-DA (Fig. 7) was able to expand the difference among categories by finding the optimal location, with improved predictability, augmented efficiency of screening biomarkers. OPLS-DA (Fig.

8) removed noise on the basis of the signal as a quadrature filter. The models above showed good degree of fit (R^2 (CUM) > 0.6) and predictability (Q^2 (CUM) > 0.5). In addition, samples were well separated through t1 or t2 dimensional model in either positive or negative ion mode which mean that GPLs in the different state.

3.5. Identifying components with high variations

For U-HPLC/Q-TOF-MS with huge three-dimensional data matrix, biomarkers are commonly screened by combining chemometrics. In a PLS-DA model, the GPLs components with VIP values exceeding 1.0 significantly affect separation as potentially eligible biomarkers [31]. In order to obtain more information on the differences between five groups, it was selected to identify potential biomarkers. Simultaneously, all of the above components should satisfy the critical t-value at 95% confidence level. Finally, the major contributing metabolite components were identified as potential biomarkers (Table 2).

In our study, searching for the potential biomarkers was used to study the relationship between GPLs and inflammation. Previous studies have shown that AA exceeded rapidly in the inflammatory cells. After de novo synthesis, it is introduced into phospholipid molecular species largely, through remodeling pathways by acyltransferases and transacylases [32]. Among the 10 potential biomarkers were including PCs and PEs which are mainly related to fatty acid metabolism and GPLs metabolism. PCs, as the most abundant GPL components in most cells, are the major constitutes of potential biomarkers. They form a stable matrix for (intra)cellular membranes based on the geometrical shape and physico-chemical properties [33]. L. M. Lichtenberger et al. reported that they exerted anti-inflammatory effects on cells [34]. When added to cells, PCs are integrated into the membrane and changes the embedded binding and signaling processes, and inflammatory response [35]. Concretely, lysoPL acyltransferase-driven reacylation pathway is dominant in unstimulated cells and the levels of AA (20:4) are kept low. When cells are stimulated, agonist-activated PLA₂ triggers the accumulation of AA which is then available for eicosanoids synthesis (prostaglandins, thromboxane, lipoxins,

leukotrienes, hydroxyeicosatetraenoic acids, endocannabinoids, and platelet activating factor). However, AA is still significantly reacylated in stimulated cells. The acyltransferase initially incorporates AA into PCs slowly by CoA-independent transacylases to other lysoPL such as 1-alkenyl-2-lysoPE [36-37]. In most of previous studies, altered PCs were associated to inflammatory condition and AA is released from membrane phospholipids [38].

PCs are a complex mixture of molecular species that have a constant aminoalcohol in their polar head, and fatty acyls of different acyl chain lengths, position, degrees of saturation. Commonly, the saturated chain existed at the sn-1 chain and the unsaturated chain at the sn-2 chain [39]. Fatty acid species at the sn-2 position are also influenced by fatty acids at the sn-1 position [40]. Acyl chain exchange might induce GPLs remodel and also affect the biological function of cell. With cell in different state, the percentage of different acyl chains also changed synchronous [41]. Different to other lipidomics, altered PCs have no consistency change in the 10 potential biomarkers which were contained the diacylglycerol and associated with inflammatory response [42]. Comparing with group Y, PC (16:0/18:1) and PC (18:0/18:1) were subjected to the same changes, the content was increased when cells were stimulated by KLA and decreased after drug treatment. The finding of these biomarkers not only makes us know the change of GPLs metabolism in inflammation better, but also provides an effective method to evaluate better the state of the disease and treat the disease in clinical practice. However, the mechanism of GPLs changing during the inflammation is not very clear, and further work is needed to better understand in the future.

4. Conclusion

Metabolic variations of GPLs in RAW264.7 cells were investigated using ¹H-NMR and U-HPLC-based metabolomics. ¹H-NMR spectroscopy was combined with multivariate data analysis to analyze GPLs and to create a simple, fast and non-destructive method to evaluate the effect of anti-inflammatory drugs on total GPLs. 58 GPLs were identified, and 19 components were firstly identified in this study compared with our previous results by U-HPLC/Q-TOF-MS. Coupled with chemometrics, 10 potential

biomarkers as the potential vital biomarkers were proved, of which PC (16:0/18:1) and PC (18:0/18:1) in three drug-induced groups changed consistently. In summary, the changes of total GPLs in RAW264.7 cells was well detected by combining ^1H -NMR spectroscopy with U-HPLC/Q-TOF-MS. Analyzing the changes among GPLs in different inflammation states helps select new anti-inflammatory drugs and provides a new way for evaluating inflammation diagnosis and prognosis.

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TABLE AND FIGURE CAPTIONS

Table 1. The identified GPLs information.

Table 2. Identified potential biomarkers.

Figure 1. ^1H -NMR metabolic spectrum of GPLs of normal RAW264.7 cells.

Figure 2. ^1H -NMR metabolic spectra of GPLs. K: GPLs information of blank group; Y: GPLs information of inflammation group; S: GPLs information of diclofenac sodium group; N: GPLs information of nimesulide group; M: GPLs information of meloxicam group.

Figure 3. Score plots of PLS-DA for blank group, diclofenac sodium group versus inflammation group (A), blank group, nimesulide group versus inflammation group (B) and blank group, meloxicam group versus the inflammation group (C) from ^1H -NMR spectroscopy. K: blank group; Y: inflammation group; S: diclofenac sodium group; N: nimesulide group; M: meloxicam group. All of the groups have suitable R^2X , R^2Y and Q^2 values. A: R^2X (cum) = 0.931, R^2Y (cum) = 0.958, Q^2 (cum) = 0.777, B: R^2X (cum) = 0.860, R^2Y (cum) = 0.698, Q^2 (cum) = 0.634, C: R^2X (cum) = 0.900, R^2Y (cum) = 0.723, Q^2 (cum) = 0.585.

Figure 4. OPLS-DA score plots (Left) and S-plot (Right) for blank group, diclofenac sodium group versus inflammation group (A and B), blank group, nimesulide group versus inflammation group (C and D) and blank group, meloxicam group versus the inflammation group (E and F) from ^1H -NMR spectroscopy. K: blank group; Y: inflammation group; S: diclofenac sodium group; N: nimesulide group; M: meloxicam group. All of the groups have suitable R^2X , R^2Y and Q^2 values. A: R^2X (cum) = 0.931, R^2Y (cum) = 0.958, Q^2 (cum) = 0.570, C: R^2X (cum) = 0.860, R^2Y (cum) = 0.698, Q^2 (cum) = 0.601, E: R^2X (cum) = 0.898, R^2Y (cum) = 0.724, Q^2 (cum) = 0.541.

Figure 5. The GPLs metabolism profiling of UPLC-Q-TOF-MS in positive (left) and negative (right) ion modes were detected for five groups as follows: blank group (K), inflammation group (Y), nimesulide group (N), diclofenac sodium group (S) and meloxicam group (M).

Figure 6. Score plots of PCA for blank group, diclofenac sodium group versus inflammation group in ESI (+) (A) and ESI (-) (B), blank group, meloxicam group versus inflammation group in ESI (+) (C) and ESI (-) (D) and blank group, nimesulide group versus inflammation group in ESI (+) (E) and ESI (-) (F) from U-HPLC/Q-TOF-MS. K: blank group; Y: inflammation group; S: diclofenac sodium group; N: nimesulide group; M: meloxicam group. All of the groups have suitable R^2X , R^2Y and Q^2 values. A: R^2X (cum) = 0.673, R^2Y (cum) = 0.848, Q^2 (cum) = 0.622, B: R^2X (cum) = 0.716, R^2Y (cum) = 0.833, Q^2 (cum) = 0.584, C: R^2X (cum) = 0.665, R^2Y (cum) = 0.851, Q^2 (cum) = 0.514, D: R^2X (cum) = 0.757, R^2Y (cum) = 0.840, Q^2 (cum) = 0.587, E: R^2X (cum) = 0.714, R^2Y (cum) = 0.841, Q^2 (cum) = 0.611, F: R^2X (cum) = 0.687, R^2Y (cum) = 0.863, Q^2 (cum) = 0.630.

Figure 7. Score plots of PLS-DA for blank group, diclofenac sodium group versus inflammation group in ESI (+) (A) and ESI (-) (B), blank group, meloxicam group versus inflammation group in ESI (+) (C) and ESI (-) (D) and blank group, nimesulide group versus inflammation group in ESI (+) (E) and ESI (-) (F) from U-HPLC/Q-TOF-MS. K: blank group; Y: inflammation group; S: diclofenac sodium group; N: nimesulide group; M: meloxicam group. All of the groups have suitable R^2X , R^2Y and Q^2 values. A: R^2X (cum) = 0.673, R^2Y (cum) = 0.845, Q^2 (cum) = 0.772, B: R^2X (cum) = 0.727, R^2Y (cum) = 0.875, Q^2 (cum) = 0.653, C: R^2X (cum) = 0.666, R^2Y (cum) = 0.850, Q^2 (cum) = 0.785, D: R^2X (cum) = 0.818, R^2Y (cum) = 0.837, Q^2 (cum) = 0.771, E: R^2X (cum) = 0.714, R^2Y (cum) = 0.841, Q^2 (cum) = 0.881, F: R^2X (cum) = 0.687, R^2Y (cum) = 0.863, Q^2 (cum) = 0.852.

Figure 8. Score plots of OPLS-DA for blank group, diclofenac sodium group versus inflammation group in ESI (+) (A) and ESI (-) (B), blank group, meloxicam group versus inflammation group in ESI (+) (C) and ESI (-) (D) and blank group, nimesulide group versus inflammation group in ESI (+) (E) and ESI (-) (F) from U-HPLC/Q-TOF-MS. K: blank group; Y: inflammation group; S: diclofenac sodium group; N: nimesulide group; M: meloxicam group. All of the groups have suitable R^2X , R^2Y and Q^2 values. A: R^2X (cum) = 0.697, R^2Y (cum) = 0.856, Q^2 (cum) = 0.625, B: R^2X (cum) = 0.803, R^2Y (cum) = 0.922, Q^2 (cum) = 0.620, C: R^2X (cum) = 0.685, R^2Y (cum) = 0.862, Q^2 (cum) =

0.688, D: R^2X (cum) = 0.837, R^2Y (cum) = 0.878, Q^2 (cum) = 0.715, E: R^2X (cum) = 0.729, R^2Y (cum) = 0.861, Q^2 (cum) = 0.698, F: R^2X (cum) = 0.744, R^2Y (cum) = 0.890, Q^2 (cum) = 0.732.

Table 1

The identified GPLs information

Peak no.	t _R (min)	Selected ion	Measured mass (<i>m/z</i>)	Calc.mass (<i>m/z</i>)	ppm	Formula	ESI-MS/MS (<i>m/z</i>)	Identification
1*	1.58	[M+H] ⁺	468.3087	468.3090	-0.6	C22H47NO7P	468 [M+H] ⁺ , 450 [M+H-H ₂ O] ⁺ , 391 [M+H-H ₂ O-59] ⁺ , 285 [M-PO ₄ CH ₂ CH ₂ N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (14:0/0:0)
2	1.17	[M+H] ⁺	466.2938	466.2934	0.9	C22H45NO7P	466 [M+H] ⁺ , 448 [M+H-H ₂ O] ⁺ , 283 [M-PO ₄ CH ₂ CH ₂ N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (14:1/0:0)
		[M-H+HCOOH] ⁻	510.2844	510.2832	2.4	C23H45NO9P	450 [M-CH ₃] ⁻ , 225 [R ₁ COO] ⁻ , 168 [C ₄ H ₁₁ O ₄ NP] ⁻	
3	1.77	[M+H] ⁺	494.3226	494.3247	-4.2	C24H49NO7P	494 [M+H] ⁺ , 476 [M+H-H ₂ O] ⁺ , 417 [M+H-H ₂ O-N(CH ₃) ₃] ⁺ , 311 [M-PO ₄ CH ₂ CH ₂ N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (16:1/0:0)
		[M-H+HCOOH] ⁻	538.3170	538.3145	4.6	C26H51NO9P	478 [M-CH ₃] ⁻ , 253 [R ₁ COO] ⁻ , 168 [C ₄ H ₁₁ O ₄ NP] ⁻	
4*	1.82	[M+H] ⁺	532.3412	532.3403	1.7	C27H51NO7P	532 [M+H] ⁺ , 514 [M+H-H ₂ O] ⁺ , 349 [M-PO ₄ CH ₂ CH ₂ N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (19:3/0:0)
5*	1.85	[M+H] ⁺	544.3416	544.3403	2.4	C28H51NO7P	544 [M+H] ⁺ , 526 [M+H-H ₂ O] ⁺ , 467 [M+H-H ₂ O-59] ⁺ , 361 [M-PO ₄ CH ₂ CH ₂ N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (0:0/20:4)
		[M-H+HCOOH] ⁻	588.3298	588.3301	-0.5	C29H51NO9P	528 [M-CH ₃] ⁻ , 303 [R ₂ COO] ⁻ , 168 [C ₄ H ₁₁ O ₄ NP] ⁻	
		[M+H] ⁺	502.2953	502.2934	3.8	C25H45NO7P	502 [M+H] ⁺ , 361 [M-C ₂ H ₇ O ₄ NP] ⁺	
6	1.88	[M-H] ⁻	500.2793	500.2777	3.2	C25H43NO7P	303 [R ₁ COO] ⁻ , 259, 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (20:4/0:0)
7	1.94	[M-H] ⁻	450.2609	450.2621	-2.7	C21H41NO7P	450 [M-H] ⁻ , 253 [R ₁ COO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (16:1/0:0)
8*	2.06	[M+H] ⁺	520.3382	520.3403	-4.0	C26H51NO7P	520 [M+H] ⁺ , 502 [M+H-H ₂ O] ⁺ , 443 [M+H-H ₂ O-N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (18:2/0:0)
		[M+H] ⁺	528.3080	528.3090	-1.9	C27H47NO7P	528 [M+H] ⁺ , 387 [M-C ₂ H ₇ O ₄ NP] ⁺	
9	2.07	[M-H] ⁻	526.2953	526.2934	3.6	C27H45NO7P	526 [M-H] ⁻ , 329 [R ₁ COO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (22:5/0:0)
10	2.21	[M+H] ⁺	508.3393	508.3403	-2.0	C25H51NO7P	508 [M+H] ⁺ , 490 [M+H-H ₂ O] ⁺ , 431 [M+H-H ₂ O-N(CH ₃) ₃] ⁺ , 324 [M-PO ₄ CH ₂ CH ₂ N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (17:1/0:0)
11*	2.21	[M-H] ⁻	476.2778	476.2777	0.2	C23H43NO7P	450 [M-H] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (18:2/0:0)
12	2.31	[M-H] ⁻	502.2921	502.2934	-2.6	C25H45NO7P	305 [R ₂ COO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (0:0/20:3)
13	2.38	[M+H] ⁺	496.3390	496.3403	-2.6	C24H51NO7P	478 [M+H-H ₂ O] ⁺ , 313 [M-PO ₄ CH ₂ CH ₂ N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (0:0/16:0)
		[M-H+HCOOH] ⁻	540.3331	540.3301	5.6	C25H51NO9P	480 [M-CH ₃] ⁻ , 255 [RCOO] ⁻ , 168 [C ₄ H ₁₁ O ₄ NP] ⁻	
14*	2.42	[M+H] ⁺	508.3409	508.3403	1.2	C25H51NO7P	508 [M+H] ⁺ , 490 [M+H-H ₂ O] ⁺ , 431 [M+H-H ₂ O-N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (17:1/0:0)
15	2.63	[M+H] ⁺	522.3550	522.3560	-1.9	C26H53NO7P	504 [M+H-H ₂ O] ⁺ , 445 [M+H-H ₂ O-59] ⁺ , 339 [M-PO ₄ CH ₂ CH ₂ N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (0:0/18:1)
		[M-H+HCOOH] ⁻	566.3434	566.3458	-4.2	C27H53NO9P	506 [M-CH ₃] ⁻ , 281 [RCOO] ⁻ , 168 [C ₄ H ₁₁ O ₄ NP] ⁻	

Peak no.	t _R (min)	Selected ion	Measured mass (<i>m/z</i>)	Calc.mass (<i>m/z</i>)	ppm	Formula	ESI-MS/MS (<i>m/z</i>)	Identification
16	2.63	[M+H] ⁺	496.3406	496.3403	0.6	C24H51NO7P	496 [M+H] ⁺ , 478 [M+H-H ₂ O] ⁺ , 419 [M+H-H ₂ O-59] ⁺ , 313 [M-PO ₄ CH ₂ CH ₂ N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (16:0/0:0)
		[M-H+HCOOH] ⁻	540.3348	540.3301	8.7	C25H51NO9P	480 [M-CH ₃] ⁻ , 255 [RCOO] ⁻ , 224 [M-CH ₃ -RCOO-] ⁻ , 153 [C ₃ H ₆ O ₅ P] ⁻	
17	2.65	[M-H] ⁻	502.2943	502.2934	1.8	C25H45NO7P	305 [R ₂ COO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (20:3/0:0)
18	2.75	[M+H] ⁺	480.3087	480.3090	-0.6	C23H47NO7P	339 [M-C ₂ H ₇ O ₄ NP] ⁺	
		[M-H] ⁻	478.2960	478.2934	5.4	C23H45NO7P	281 [RCOO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (0:0/18:1)
19*	2.82	[M+H] ⁺	544.3376	544.3403	-5.0	C28H51NO7P	544 [M+H] ⁺ , 526 [M+H-H ₂ O] ⁺ , 467 [M+H-H ₂ O-59] ⁺	PC (20:4/0:0)
20	2.82	[M+H] ⁺	522.3568	522.3560	1.5	C26H53NO7P	361 [M-PO ₄ CH ₂ CH ₂ N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺ , 522 [M+H] ⁺ , 504 [M+H-H ₂ O] ⁺ , 445 [M+H-H ₂ O-59] ⁺ , 339 [M-PO ₄ CH ₂ CH ₂ N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (18:1/0:0)
		[M-H+HCOOH] ⁻	566.3476	566.3458	3.2	C27H53NO9P	506 [M-CH ₃] ⁻ , 281 [RCOO] ⁻ , 168 [C ₄ H ₁₁ O ₄ NP] ⁻	
21*	2.84	[M-H] ⁻	452.2752	452.2777	-5.5	C21H43NO7P	255 [R ₁ COO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (16:0/0:0)
22*	2.86	[M-H] ⁻	566.3434	566.3458	-4.2	C27H53NO9P	weak	PS (21:0/0:0)
		[M+H] ⁺	508.3418	508.3403	3.0	C25H51NO7P	508 [M+H] ⁺ , 367 [M-C ₂ H ₇ O ₄ NP] ⁺	
23	2.86	[M-H] ⁻	506.3267	506.3247	4.0	C25H49NO7P	309 [R ₁ COO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (20:1/0:0)
24*	2.95	[M-H] ⁻	528.3100	528.3090	1.9	C27H47NO7P	331 [R ₁ COO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (22:4/0:0)
25	2.97	[M+H] ⁺	480.3100	480.3090	2.1	C23H47NO7P	480 [M+H] ⁺ , 339 [M-C ₂ H ₇ O ₄ NP] ⁺	
		[M-H] ⁻	478.2958	478.2934	5.0	C23H45NO7P	478 [M-H] ⁻ , 281 [RCOO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 153 [C ₃ H ₆ O ₅ P] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (18:1/0:0)
26*	3.28	[M+H] ⁺	508.3752	508.3767	-3.0	C26H55NO6P	508 [M+H] ⁺ , 490 [M+H-H ₂ O] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (P-18:0/0:0) or PC (O-18:1/0:0)
27*	3.45	[M-H] ⁻	435.2515	435.2512	0.7	C21H40O7P	435 [M-H] ⁻ , 153 [C ₃ H ₆ O ₅ P] ⁻	PA (18:1/0:0) or PA (0:0/18:1)
28*	3.75	[M-H] ⁻	508.3407	508.3403	0.8	C25H51NO7P	311 [R ₁ COO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (20:0/0:0)
29	3.78	[M+H] ⁺	524.3698	524.3716	-3.4	C26H55NO7P	524 [M+H] ⁺ , 506 [M+H-H ₂ O] ⁺ , 447 [M+H-H ₂ O-59] ⁺ , 341 [M-PO ₄ CH ₂ CH ₂ N(CH ₃) ₃] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (18:0/0:0)
		[M-H+HCOOH] ⁻	568.3634	568.3614	3.5	C27H55NO9P	508 [M-CH ₃] ⁻ , 283 [RCOO] ⁻ , 168 [C ₄ H ₁₁ O ₄ NP] ⁻	
		[M+H] ⁺	482.3275	482.3247	5.8	C23H49NO7P	482 [M+H] ⁺ , 341 [M-C ₂ H ₇ O ₄ NP] ⁺	
30	3.78	[M-H] ⁻	480.3110	480.3090	4.2	C23H47NO7P	283 [R ₁ COO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 153 [C ₃ H ₆ O ₅ P] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (0:0/18:0)
31*	3.84	[M+H] ⁺	550.3873	550.3873	0.0	C28H57NO7P	550 [M+H] ⁺ , 532 [M+H-H ₂ O] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (20:1/0:0)
		[M-H+HCOOH] ⁻	594.3802	594.3771	5.2	C29H57NO9P	548 [M-H] ⁻ , 309 [R ₁ COO] ⁻	
32	3.93	[M+H] ⁺	482.3263	482.3247	3.3	C23H49NO7P	482 [M+H] ⁺ , 341 [M-C ₂ H ₇ O ₄ NP] ⁺	PE (18:0/0:0)

Peak no.	t _R (min)	Selected ion	Measured mass (<i>m/z</i>)	Calc.mass (<i>m/z</i>)	ppm	Formula	ESI-MS/MS (<i>m/z</i>)	Identification
		[M-H] ⁻	480.3076	480.3090	-2.9	C23H47NO7P	281 [R ₁ COO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 153 [C ₃ H ₆ O ₅ P] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	
33	4.73	[M-H] ⁻	534.3566	534.3560	1.1	C27H53NO7P	534 [M-H] ⁻ , 337 [R ₁ COO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (22:1/0:0)
34	6.50	[M-H] ⁻	819.5147	819.5176	-3.5	C46H76O10P	819 [M-H] ⁻ , 327 [R ₁ COO] ⁻ , 281 [R ₂ COO] ⁻	PG (22:6/18:1)
35	6.79	[M-H] ⁻	745.5018	745.5020	-0.3	C40H74O10P	745 [M-H] ⁻ , 671 [M-H-74] ⁻ , 281 [R ₂ COO] ⁻ , 253 [R ₁ COO] ⁻	PG (18:1/16:1)
		[M+H] ⁺	730.5367	730.5387	-2.7	C40H77NO8P	730 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
36	7.64	[M-H+HCOOH] ⁻	774.5317	774.5285	4.1	C41H77NO10P	714 [M-CH ₃] ⁻ , 460 [M-CH ₃ -R ₂ COOH] ⁻ , 253 [R ₁ COO] ⁻	PC (16:1/16:1)
37	8.29	[M-H] ⁻	773.5361	773.5333	3.6	C42H78O10P	773 [M-H] ⁻ , 417 [M-R ₂ COOH-74] ⁻ , 281 [RCOO] ⁻ , 227 [C ₆ H ₁₂ O ₇ P] ⁻ , 153 [C ₃ H ₆ O ₅ P] ⁻	PG (18:1/18:1)
		[M+H] ⁺	706.5400	706.5387	1.8	C38H77NO8P	706 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
38	8.85	[M-H+HCOOH] ⁻	750.5330	750.5285	6.0	C39H77NO10P	255 [R ₂ COO] ⁻ , 227 [R ₁ COO] ⁻ , 168 [C ₄ H ₁₁ O ₄ NP] ⁻	PC (14:0/16:0)
		[M+H] ⁺	808.5847	808.5856	-1.1	C46H83NO8P	808 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
39*	8.906	[M-H+HCOOH] ⁻	852.5774	852.5755	2.2	C47H83NO10P	792 [M-CH ₃] ⁻ , 303 [R ₁ COO] ⁻ , 281 [R ₂ COO] ⁻	PC (20:4/18:1)
		[M+H] ⁺	732.5536	732.5543	-1.0	C40H79NO8P	732 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
40	8.92	[M-H+HCOOH] ⁻	776.5481	776.5442	5.0	C41H79NO10P	776 [M-H+HCOOH] ⁻ , 716 [M-CH ₃] ⁻ , 253 [R ₂ COO] ⁻], 227 [R ₁ COO] ⁻ , 168 [C ₄ H ₁₁ O ₄ NP] ⁻	PC (14:0/18:1)
		[M+H] ⁺	758.5675	758.5700	-3.3	C42H81NO8P	758 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
41	9.02	[M-H+HCOOH] ⁻	802.5643	802.5598	5.6	C43H81NO10P	742 [M-CH ₃] ⁻ , 281 [R ₁ COO] ⁻ , 253 [R ₂ COO] ⁻	PC (18:1/16:1)..
								PC (16:0/20:3) or PC (18:1/18:2)...
42	9.41	[M+H] ⁺	784.5837	784.5856	-2.4	C44H83NO8P	784 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
		[M+H] ⁺	810.6039	810.6013	3.2	C46H85NO8P	810 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
43	9.92	[M-H+HCOOH] ⁻	854.5913	854.5911	0.2	C47H85NO10P	794 [M-CH ₃] ⁻ , 305 [R ₁ COO] ⁻ , 281 [R ₂ COO] ⁻	PC (18:1/20:3)
		[M+H] ⁺	746.5729	746.5700	3.9	C41H81NO8P	746 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
44	10.05	[M-H+HCOOH] ⁻	790.5649	790.5598	6.5	C42H81NO10P	804 [M-H+CH ₃ COOH] ⁻ , 730 [M-CH ₃] ⁻ , 241 [R ₁ COO] ⁻ , 281 [R ₂ COO] ⁻	PC (15:0/18:1)
		[M+H] ⁺	746.5714	746.5700	1.9	C41H81NO8P	746 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
45	10.63	[M-H+HCOOH] ⁻	790.5609	790.5598	1.4	C42H81NO10P	804 [M-H+CH ₃ COOH] ⁻ , 730 [M-CH ₃] ⁻ , 267 [R ₂ COO] ⁻ , 255 [R ₁ COO] ⁻ .	PC (16:0/17:1)
		[M+H] ⁺	810.6027	810.6013	1.7	C46H85NO8P	810 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
46*	10.72	[M-H+HCOOH] ⁻	854.5918	854.5911	0.8	C47H85NO10P	794 [M-CH ₃] ⁻ , 303 [R ₁ COO] ⁻ , 283 [R ₂ COO] ⁻	PC (20:4/18:0)
		[M+H] ⁺	734.5703	734.5700	0.4	C40H81NO8P	734 [M+H] ⁺ , 496 [M+H-RCOOH] ⁺ , 478 [M+H- RCOOH-H ₂ O] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (16:0/16:0)
		[M-H+HCOOH] ⁻	778.5610	778.5598	1.5	C41H81NO10P	718 [M-CH ₃] ⁻ , 255 [R ₂ COO] ⁻	
		[M+H] ⁺	760.5857	760.5856	0.1	C42H83NO8P	760 [M+H] ⁺ , 522 [M+H-C ₁₄ H ₂₉ CHC=O] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺ , 496 [M+H-C ₁₆ H ₃₁ CHC=O] ⁺ , 478 [M+H-C ₁₇ H ₃₃ COOH] ⁺	PC (16:0/18:1)
48	11.39	[M-H+HCOOH] ⁻	804.5779	804.5755	3.0	C43H83NO10P	744 [M-CH ₃] ⁻ , 462 [M-CH ₃ -R ₂ COOH] ⁻ , 281 [R ₂ COO] ⁻ , 255 [R ₁ COO] ⁻ , 168 [C ₄ H ₁₁ O ₄ NP] ⁻	
49	11.40	[M+H] ⁺	786.6039	786.6013	3.3	C44H85NO8P	786 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (18:1/18:1)

Peak no.	t _R (min)	Selected ion	Measured mass (<i>m/z</i>)	Calc.mass (<i>m/z</i>)	ppm	Formula	ESI-MS/MS (<i>m/z</i>)	Identification
		[M-H+HCOOH] ⁻	830.5917	830.5911	0.7	C45H85NO10P	770 [M-CH ₃] ⁻ , 488 [M-15-R ₂ COOH] ⁻ , 281 [R ₂ COO] ⁻ , 168 [C ₄ H ₁₁ O ₄ NP] ⁻	
50	11.77	[M-H] ⁻	766.5371	766.5387	-2.1	C43H77NO8P	766 [M-H] ⁻ , 462 [M-H-R ₂ COOH] ⁻ , 303 [R ₂ COO] ⁻ , 283 [R ₁ COO] ⁻	PE (18:0/20:4)
		[M+H] ⁺	746.6077	746.6064	1.7	C42H85NO7P	746 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
51	13.00	[M-H+HCOOH] ⁻	790.5964	790.5962	0.3	C43H85NO9P	730 [M-CH ₃] ⁻ , 448 [M-CH ₃ - R ₂ COO] ⁻ , 281 [R ₂ COO] ⁻	PC (O-16:0/18:1)
52*	13.08	[M+H] ⁺	746.6082	746.6064	2.4	C42H85NO7P	746 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (O-18:1/16:0)
	9	[M-H+HCOOH] ⁻	790.5956	790.5962	-0.8	C43H85NO9P	730 [M-CH ₃] ⁻ , 281 [R ₂ COO] ⁻ , 255 [R ₂ COO] ⁻	
53*	13.10	[M+H] ⁺	772.6229	772.6220	1.2	C44H87NO7P	772 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (18:1/P-18:0)
	8	[M-H+HCOOH] ⁻	816.6129	816.6118	1.3	C45H87NO9P	756 [M-CH ₃] ⁻ , 492, 281 [R ₁ COO] ⁻	
54*	14.31	[M+H] ⁺	814.6353	814.6326	3.3	C46H89NO8P	814 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (18:1/20:1)
	5	[M-H+HCOOH] ⁻	858.6230	858.6224	0.7	C47H89NO10P	798 [M-CH ₃] ⁻ , 281 [R ₁ COO] ⁻ , 309 [R ₂ COO] ⁻	
55	14.30	[M+H] ⁺	788.6176	788.6169	0.9	C44H87NO8P	788 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	PC (18:0/18:1)
		[M-H+HCOOH] ⁻	832.6047	832.6068	-2.5	C45H87NO10P	283 [R ₁ COO] ⁻ , 281 [R ₂ COO] ⁻	
		[M+H] ⁺	746.5730	746.5700	4.0	C41H81NO8P	746 [M+H] ⁺ , 605 [M-C ₂ H ₇ O ₄ NP] ⁺	
56	15.08	[M-H] ⁻	744.5526	744.5543	-2.3	C41H79NO8P	744 [M-H] ⁻ , 462 [M-H- R ₂ COO] ⁻ , 281 [R ₂ COO] ⁻ , 283 [R ₁ COO] ⁻ , 196 [C ₅ H ₁₁ O ₅ NP] ⁻ , 140 [C ₂ H ₇ O ₄ NP] ⁻	PE (18:0/18:1)
		[M+H] ⁺	748.6216	748.6220	-0.5	C42H87NO7P	748 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
57	16.07	[M-H+HCOOH] ⁻	792.6122	792.6118	0.5	C43H87NO9P	746 [M-H] ⁻ , 283 [R ₁ COO] ⁻ , 255 [R ₂ COO] ⁻ , 153 [C ₃ H ₆ O ₅ P] ⁻	PC (18:0/16:0)
		[M+H] ⁺	774.6395	774.6377	2.3	C44H89NO7P	774 [M+H] ⁺ , 184 [C ₅ H ₁₅ O ₄ NP] ⁺	
58	16.11	[M-H+HCOOH] ⁻	818.6287	818.6275	1.5	C45H89NO9P	758 [M-CH ₃] ⁻ , 309 [R ₁ COO] ⁻ , 255 [R ₂ COO] ⁻	PC (P-20:0/16:0)

* explains the firstly identified components in this study.

Table 2

Identified potential biomarkers

No.	Identification	Change trend			
		K vs. Y	M vs. Y	N vs. Y	S vs. Y
1	PC (14:0/0:0)	↓	—	↑	↑
2	PC (16:1/0:0)	↓	—	↑	↑
3	PC (0:0/20:4)	↓	—	—	↑*
4	PC (18:2/0:0)	↓	—	↑	↑
5	PC (20:4/18:0)	—	—	↑	↑
6	PC (16:0/18:1)	↑	↓	↓*	↓*
7	PC (18:1/P-18:0)	—	↓	—	↓
8	PC (18:0/18:1)	↑	↓	↓	↓
9	PE (18:0/18:1)	—	↑	—	↑
10	PC (P-20:0/16:0)	—	↓	↓	—

↑ indicates increase; ↓ indicates decrease; — indicates no significant changes.

* indicates significant change ($p < 0.01$), the other indicates significant change ($p < 0.05$).

K: blank group; Y: inflammation group; M: meloxicam group; N: nimesulide group; S: diclofenac sodium group.

Fig. 1 ^1H -NMR metabolic spectrum of GPLs of normal RAW264.7 cells

Fig. 2 ^1H -NMR metabolic spectra of GPLs. K: GPLs information of blank group; Y: GPLs information of inflammation group; S: GPLs information of diclofenac sodium group; N: GPLs information of nimesulide group; M: GPLs information of meloxicam group.

Fig. 3 Score plots of PLS-DA for blank group, diclofenac sodium group versus inflammation group (A), blank group, nimesulide group versus inflammation group (B) and blank group, meloxicam group versus the inflammation group (C) from ¹H-NMR spectroscopy. K: blank group; Y: inflammation group; S: diclofenac sodium group; N: nimesulide group; M: meloxicam group. All of the groups have suitable R^2X , R^2Y and Q^2 values. A: R^2X (cum) = 0.931, R^2Y (cum) = 0.958, Q^2 (cum) = 0.777, B: R^2X (cum) = 0.860, R^2Y (cum) = 0.698, Q^2 (cum) = 0.634, C: R^2X (cum) = 0.900, R^2Y (cum) = 0.723, Q^2 (cum) = 0.585.

Fig. 4 OPLS-DA score plots (Left) and S-plot (Right) for blank group, diclofenac sodium group versus inflammation group (A and B), blank group, nimesulide group versus inflammation group (C and D) and blank group, meloxicam group versus the inflammation group (E and F) from ^1H -NMR spectroscopy. K: blank group; Y: inflammation group; S: diclofenac sodium group; N: nimesulide group; M: meloxicam group. All of the groups have suitable R^2X , R^2Y and Q^2 values. A: R^2X (cum) = 0.931, R^2Y (cum) = 0.958, Q^2 (cum) = 0.570, C: R^2X (cum) = 0.860, R^2Y (cum) = 0.698, Q^2 (cum) = 0.601, E: R^2X (cum) = 0.898, R^2Y (cum) = 0.724, Q^2 (cum) = 0.541.

Fig. 5 The GPLs metabolism profiling of UPLC-Q-TOF-MS in positive (left) and negative (right) ion modes were detected for five groups as follows: blank group (K), inflammation group (Y), nimesulide group (N), diclofenac sodium group (S) and meloxicam group (M).

Fig. 6 Score plots of PCA for blank group, diclofenac sodium group versus inflammation group in ESI (+) (A) and ESI (-) (B), blank group, meloxicam group versus inflammation group in ESI (+) (C) and ESI (-) (D) and blank group, nimesulide group versus inflammation group in ESI (+) (E) and ESI (-) (F) from U-HPLC/Q-TOF-MS. K: blank group; Y: inflammation group; S: diclofenac sodium group; N: nimesulide group; M: meloxicam group. All of the groups have suitable R^2X , R^2Y and Q^2 values. A: R^2X (cum) = 0.673, R^2Y (cum) = 0.848, Q^2 (cum) = 0.622, B: R^2X (cum) = 0.716, R^2Y (cum) = 0.833, Q^2 (cum) = 0.584, C: R^2X (cum) = 0.665, R^2Y (cum) = 0.851, Q^2 (cum) = 0.514, D: R^2X (cum) = 0.757, R^2Y (cum) = 0.840, Q^2 (cum) = 0.587, E: R^2X (cum) = 0.714, R^2Y (cum) = 0.841, Q^2 (cum) = 0.611, F: R^2X (cum) = 0.687, R^2Y (cum) = 0.863, Q^2 (cum) = 0.630.

Fig. 7 Score plots of PLS-DA for blank group, diclofenac sodium group versus inflammation group in ESI (+) (A) and ESI (-) (B), blank group, meloxicam group versus inflammation group in ESI (+) (C) and ESI (-) (D) and blank group, nimesulide group versus inflammation group in ESI (+) (E) and ESI (-) (F) from U-HPLC/Q-TOF-MS. K: blank group; Y: inflammation group; S: diclofenac sodium group; N: nimesulide group; M: meloxicam group. All of the groups have suitable R^2X , R^2Y and Q^2 values. A: R^2X (cum) = 0.673, R^2Y (cum) = 0.845, Q^2 (cum) = 0.772, B: R^2X (cum) = 0.727, R^2Y (cum) = 0.875, Q^2 (cum) = 0.653, C: R^2X (cum) = 0.666, R^2Y (cum) = 0.850, Q^2 (cum) = 0.785, D: R^2X (cum) = 0.818, R^2Y (cum) = 0.837, Q^2 (cum) = 0.771, E: R^2X (cum) = 0.714, R^2Y (cum) = 0.841, Q^2 (cum) = 0.881, F: R^2X (cum) = 0.687, R^2Y (cum) = 0.863, Q^2 (cum) = 0.852.

Fig. 8 Score plots of OPLS-DA for blank group, diclofenac sodium group versus inflammation group in ESI (+) (A) and ESI (-) (B), blank group, meloxicam group versus inflammation group in ESI (+) (C) and ESI (-) (D) and blank group, nimesulide group versus inflammation group in ESI (+) (E) and ESI (-) (F) from U-HPLC/Q-TOF-MS. K: blank group; Y: inflammation group; S: diclofenac sodium group; N: nimesulide group; M: meloxicam group. All of the groups have suitable R^2X , R^2Y and Q^2 values. A: R^2X (cum) = 0.697, R^2Y (cum) = 0.856, Q^2 (cum) = 0.625, B: R^2X (cum) = 0.803, R^2Y (cum) = 0.922, Q^2 (cum) = 0.620, C: R^2X (cum) = 0.685, R^2Y (cum) = 0.862, Q^2 (cum) = 0.688, D: R^2X (cum) = 0.837, R^2Y (cum) = 0.878, Q^2 (cum) = 0.715, E: R^2X (cum) = 0.729, R^2Y (cum) = 0.861, Q^2 (cum) = 0.698, F: R^2X (cum) = 0.744, R^2Y (cum) = 0.890, Q^2 (cum) = 0.732.