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Short Communication

PRECOLUMN DERIVATIZATION LIQUID CHROMATOGRAPHY WITH MASS SPECTROMETRY ASSAY FOR THE DETERMINATION OF GLUCOSAMINE IN SMALL VOLUME HUMAN PLASMA

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INTRODUCTION

Glucosamine is an amino-monosaccharide biosynthesized endogenously both in animals and humans by amination of glucose[1]. Beneficial pharmacologic effects were reported for glucosamine, including antiarthritic and anti-inflammatory properties and an ability to stimulate proteoglycan syntheses through chondrocytes activation[2, 3]. Vitro studies have shown that glucosamine stimulates the production of proteoglycan and increases sulfate uptake by articular cartilage[4,5]. Some studies results supported the classification of glucosamine as anti-reactive rather than anti-inflammatory, because it did not affect cyclooxygenase[6].

Glucosamine analysis has been reported in nutritional supplements[7], soils[8], and plants[9]. Assay for glucosamine along with amino acids in bacterial cell walls, glycoconjugate, soya chitin have also been described[10-12]. In animals and human, derived biological samples, radiolabeled glucosamine have been used for quantitative determining the concentration of glucosamine and its metabolites in pharmacokinetic studies. However, using radioactivity to quantitate glucosamine might potentially confound the results since the parent drug cannot be differentiated from its metabolites and/or degradation products[13,14]. A precolumn derivatization HPLC method has also been reported for the assay of glucosamine in raw materials, nutraceutial preparations and dog plasma[15] with lower limit of quantitation (LLOQ) 1.25 μg mL^{-1}. We here reported firstly a specific and sensitive method, based upon a precolumn derivatization HPLC with electrospray ionization mass spectrometry (ESI-MS), to determine glucosamine in human plasma.

EXPERIMENTAL

Apparatus

An Agilent 1100 Series LC-MS system was used to inject 5-μL aliquot of the processed samples on a 5-μm Inertsil ODS-3 (150×2.1 mm i.d.), obtained from Agilent (USA), which was kept at the temperature of 40°C. Ahead of the analysis column, a SecurityGuardTM HPLC guard cartridge system from Phenomenex (USA) was used to protect the analytical column. The mobile phase consisted of 0.5% acetic acid (Tedia, USA) solution as elution solvent A and pure methanol (Merck) as elution solvent B, was delivered at the first flow 0.3 mL min^{-1}. A gradient elution was applied in TABLE 1.

Quantification was achieved by MS detection in the positive ion mode, using ESI as an interface, with the drying gas flow at 10 L min^{-1},the drying gas temperature at 350°C, the nebulizer pressure at 50 psi, the capillary voltage at 4000 V, fragment at 100V. In the Selective Ion

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Monitoring (SIM) mode, the m/z 297.1 ion was detected for glucosamine derivative (MW= 296.3) and galactosamine derivative (MW=302.042).

TABLE 1 - Mobile phase gradient elution

t (min)	A%	B%	flow rate（mL min^{-1}）
0	80	20	0.3
8	10	90	0.3
8.01	10	90	0.4
13	10	90	0.4

Reagents

A stock solution of glucosamine was prepared by dissolving 10mg glucosamine sulfate in 10mL water (1mg/mL). Appropriate amounts of the stock solution were diluted with water to give concentration standards solutions in the range of 1-100μg mL^{-1}. These were subsequently spiked to blank plasma for the preparation of plasma standards. The aqueous solutions and plasma standards were freshly prepared prior to use.

Sample preparations and derivazitation

10μL internal standard (D(+) galactosamine hydrochloride, 30μg mL^{-1}) was added into 0.1mL human plasma. Plasma proteins were precipitated by adding 0.3mL of acetonitrile followed by vortex mixing (one minute) and centrifugation (five minutes) at 10,000×g. 0.2mL of supernatant was transferred into a 1.5mL disposable tube and 20μL derivatizing reagent (phenyl iso-thiocyanate-triethylamine-acetonitrile, 2:2:6) was added. After vortexed completely, sample was allowed to derivatization react at 60°C for 40min and 5μL was injected into the HPLC/MS system.

RESULTS AND DISCUSSION

Developing of method and selection of derivatization reagents

Considering the structure of glucosamine, it does not present a suitable chromophore, UV detector can not be used. Accordingly, MS detector might be an advantage. However, the preliminary experiment showed that the retention time of glucosamine was short on the C_{18} column for its strong polarity. Therefore, a derivatiztion step was employed to convert the target analytes into compounds facilitated HPLC analysis. In addition, the dervatization product should present characteristic mass spectra with specific fragment ions for MS detection. Derivatization reagents such as o-Phthaldialdehyde, 2, 4-Dinitrofluorobenzen that were commonly applied for amino acids were investigated. The derivative compounds of o-Phthaldialdehyde were unstable and decomposed with time. Along with some subsidiary reaction, 2, 4-dinitrofluorobenzene derivative had low sensitivity in MSD. Phenyl iso-thiocyanate obtained glucosamine derivative that had selective mass spectra. Therefore the phenyl iso-thiocyanate was chosen for derivatization step. The conditions of derivatization including the volume of derivative reagent and triethylamine (catalyzer), reaction temperature and time were optimized. It was determined that the ratio of phenyl iso-thiocyanate-triethylamine-acetonitrile was 2:2:6, react temperature 60°C and react time 40min.

Chromatography and Mass spectra

Full-scan ESI-positive mass spectra of glucosamine derivative was determined, in which protonated molecules $(MH)^+$ of glucosamine derivatizative was observed at m/z 297, corresponding

with possible derivatizative reactions shown in FIG.1. Typical chromatography was shown in FIG.2. The retention time of glucosamine derivative and galactosamine derivative (internal standard) were 6.4 and 7.1 minutes respectively. Glucosamine derivative, internal standard and endogenous plasma components were separated. The method was found to be specific for glucosamine and no interfering peaks induced by internal standard, plasma or reagents were detected.

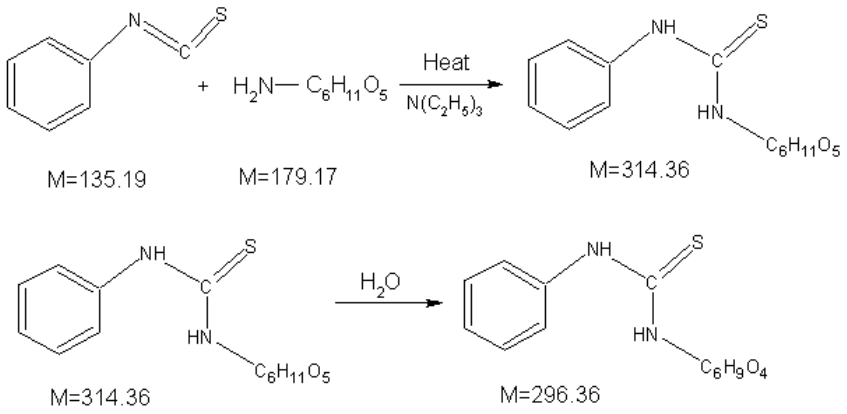

FIG. 1 - Possible derivatizative reactions

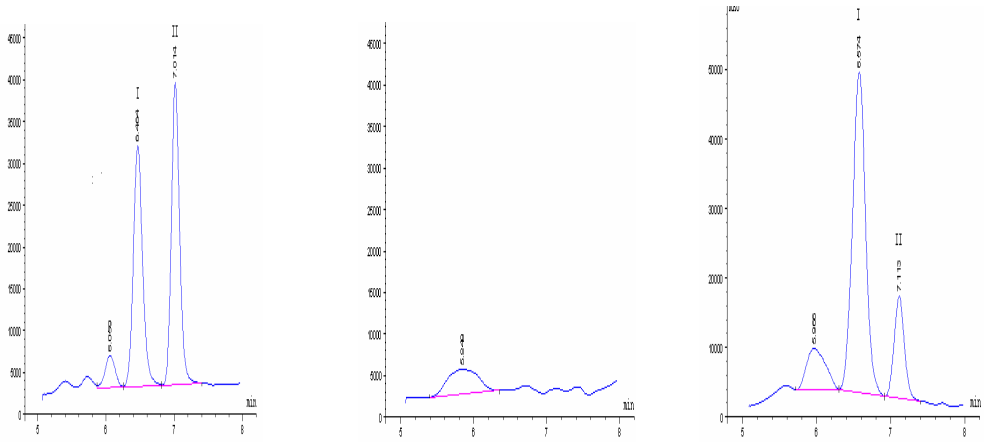

FIG. 2 - Typical chromatography for glucosamine and internal standard (IS) in spiked plasma (A), blank plasma (B) and a volunteer sample (C). Retention time of glucosamine and internal standard are 6.5 and 7.1 respectively

Calibration and quality control samples quantification

Six calibrator standards containing 0.5, 1, 2, 5, 10 and 20 μg mL^{-1} glucosamine were prepared by adding the proper amounts to drug-free human plasma samples. 10μL internal standard (30μg mL^{-1} solution) was added into each 0.1mL calibration sample. The procedure of plasma proteins precipitation and sample derivazitation were the same as described in 2.3. The calibration curve was based on the ratio of peak areas of glucosamine to internal standard versus the concentration of glucosamine added. The correlation coefficient was 0.9991 in the range of 0.5 -10μg mL^{-1} (TABLE 2).

TABLE 2- Data of standard curves of glucosamine (n=3)

Concentration(μg mL^{-1}), nominal	observed	Bias%
0.5	0.57	15.0
1	1.11	11.0
2	2.27	14.0
5	4.62	-7.6
10	9.49	-5.1
20	20.31	1.6

Accuracy and Precision

TABLE 3 showed the inter- and intra-day precision and accuracy at three concentration levels (0.5, 2, 10μg mL^{-1}). Intra-day precision was determined by analyzing parallel five samples at each concentration in a single day. Inter-day precision was performed by repeated assay of these samples over five successive days. The inter- and intra-day %R.S.D ranged from 3.09 to 8.08% and 5.59 to 6.38% respectively. The precision and accuracy data showed that this LC/MS method was consistent and reliable for the standards and quality control samples over the entire concentration range.

The lower limit of detection (signal-to-noise ratio > 3:1) of glucosamine at 0.1μg mL^{-1} was founded in spiked sample, prepared by added diluted solution to drug-free human plasma.

Stability

The stability of glucosamine was evaluated by comparing the peak area ratio of glucosamine to the internal standard. Spiked plasma samples at three concentrations (0.5, 2, 10μg mL^{-1}) were prepared. All stability test sample solutions were stored at -20°C. For each concentration, three of those stability samples were measured during 60 days. The stability of derivative was also tested at three concentrations. The freshly prepared standards were stored at room temperature over 24 hours. Both glucosamine and derivative at mentioned conditions showed good stability with RSD% less than 10%.

TABLE 3- Summary of accuracy and precision results for glucosamine at three concentration levels

Nominal concentration ($ng\ mL^{-1}$)	Intra-day (n=5) measured concentration($ng\ mL^{-1}$)		Inter-day (n=5) measured concentration($ng\ mL^{-1}$)	
	Mean	RSD %	Mean	RSD %
0.5	0.55	8.08	0.57	6.38
2	2.25	3.09	2.19	5.59
10	9.56	5.77	9.81	6.38

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