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Short communication

Determination of underivatized glucosamine in human plasma by high-performance liquid chromatography with electrochemical detection: Application to pharmacokinetic study

E. Pashkova*, A. Pirogov, A. Bendryshev, E. Ivanaynen, O. Shpigun

Moscow State University, Analytical Chemistry Department, 119991, GSP-1, Moscow, Russia

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ABSTRACT

A simple, rapid and sensitive high-performance liquid chromatography method with electrochemical detection was developed for the determination of glucosamine in human plasma. Plasma samples were analyzed after a simple two-step procedure of protein precipitation with subsequent dilution. The chromatographic separation was performed on a Carbopack™ column (3 mm × 150 mm) with a mobile phase consisting of water and 200 mM sodium hydroxide. Detection was performed electrochemically in a pulsed voltammetry mode. The limit of detection was 2.0 ng/ml, inter- and intra-day precision were less than 10%. The method was successfully applied to the investigation of the pharmacokinetics of glucosamine in healthy man volunteers.

1. Introduction

Glucosamine (2-amino-2-deoxy-D-glucose), an amino monosaccharide, is a natural compound of the glycoproteins present in connective tissues and gastrointestinal mucosal membrane, which acts as a building block of glucosaminoglycans [1]. Its chemical structure is shown in Fig. 1. Glucosamine supplements are suggested to provide symptomatic relief for osteoarthritis and related diseases [2]. Dietary supplements, based on this compound, are recommended by the Food and Drug Administration; moreover, the use of a number of glucosamine-containing pharmaceuticals for the treatment of osteoarthritis is increasing rapidly. The quantitative determination of glucosamine in biological fluids is important due to investigation of its biological role and metabolism, as well as to studying its pharmacokinetics. A number of literature methods for the determination of glucosamine were reported recently. One of the most popular techniques is liquid chromatography with mass-selective detection [3–5]. It should be noted that authors [3,4] often apply complicated and expensive tandem MS/MS technique to achieve necessary selectivity and sensitivity. Different chromatographic methods with fluorescent detection and precolumn derivatization were also described [6–9]. 9-Fluorenmethoxycarbonyl chloride (FMOC) and 8-aminopyrenesulfonic acid (APTS) were used for the derivatization. In the case of APTS method of capillary electrophoresis with laser-induced fluorescence detection was also applied successfully [9].

Method of anion-exchange chromatography with electrochemical detection was used for the analysis of the mixtures of the hydrolyzed glycosaminoglycans [10] and polysaccharides [11], galactose- and fructose derivatives [11,12]. This technique possesses high sensitivity and selectivity, moreover, it is simple enough to be used for routine analyses in clinical laboratories. In this work a simple, sensitive and selective method for the determination of glucosamine in human plasma was developed and validated. Pharmacokinetics of glucosamine in healthy male volunteers was investigated.

2. Experimental

2.1. Reagents and chemicals

D-(+)-Glucosamine (99,7%) was purchased from Sigma–Aldrich (USA). Acetonitrile (HPLC grade) was purchased from Panreac (Spain). All samples and standards were diluted with 18 MΩ water.

2.2. Apparatus and operation conditions

The analyses were performed by Dionex ion chromatography system (ICS 3000), which consisted of gradient pump with on-line mobile phase degasser, manual injector with a 50 μl sample loop, column thermostat and electrochemical detector, equipped

* Corresponding author. Tel.: +7 495 939 46 87; fax: +7 495 930 32 41.
E-mail address: e_pashkova@list.ru (E. Pashkova).

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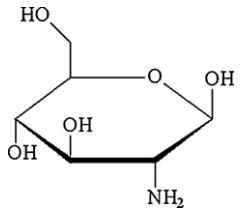

Fig. 1. The chemical structure of glucosamine.

with gold working electrode and Ag/AgCl reference electrode. The separation was performed on a Carbopack™ PA column (3 mm × 150 mm, 6.5 μm), equipped with Carbopack™ guard column (3 mm × 30 mm, 6.5 μm) in a gradient mode: solvent A – water, solvent B – 200 mM sodium hydroxide, 0–8 min A:B 95:5 (v/v), 8.1–10 min 100% B, 10.1–12 min A:B 95:5 (v/v). Flow rate was 0.5 ml/min. The potential waveform was as follows: 0–0.4 s, E = 0.1 V; 0.41–0.42 s, E = −2 V; 0.43 s, E = 0.6 V; 0.44–0.5 s, E = −0.1 V. Integration was on from 0.2 to 0.4 s. The current–voltage relationship was obtained in a 3D voltammetric mode of the analysis. Data acquisition and instrument control were performed using Chromeleon 6.80 software (Dionex, USA).

2.3. Preparation of standard solution and quality control (QC) samples

To prepare stock solution of glucosamine, 25 mg of glucosamine was dissolved in 100 ml of distilled water. The working solutions with lower concentrations (25 and 2 mg/l) were prepared daily by diluting the stock solution with water. The stock solution was stored at 4 °C. Calibration standard solutions were obtained on 5 different days by spiking 0.2 ml of blank human plasma with necessary amount of working glucosamine solution to yield concentrations of 10, 50, 100, 500, 1000 and 6000 μg/l. The quality control (QC) samples were obtained daily in the same way at low, medium and high concentration levels (10, 400 and 2000 μg/l). Standard and QC samples were pretreated in the same manner, described below.

2.4. Plasma samples preparation

200 μl of plasma sample was mixed with 400 μl of acetonitrile, the mixture was vortex mixed for 1 min and centrifuged at 16 000 rpm for 3 min. Then 500 μl of supernatant were taken and diluted with water four times. An aliquot of 50 μl was injected into the chromatographic system.

2.5. Method validation

2.5.1. Selectivity

Six different blank human plasma samples were analyzed to investigate the possible interference of endogenous compounds.

2.5.2. Linearity

To establish the linearity, a series of spiked plasma samples in the range of 10–6000 μg/l was analyzed three times. The calibration curves were calculated by using the glucosamine peak area versus nominal concentration and fitted to the equation $y = ax + b$ weighted least-squares linearity regression. The limit of detection (LOD) was calculated as the lowest concentration level resulting to a signal-to-noise ratio of 3:1, and the lower limit of quantification (LLOQ) of glucosamine was defined as the lowest concentration of the calibration curve.

2.5.3. Precision and accuracy

To determine the inter-day precision five replicates of each QC sample were analyzed on the same day. The intra-day precision was established in the same way on 3 different days. To determine the accuracy, for each QC sample the calculated concentration was compared with the theoretical.

2.5.4. Extraction recovery

The recovery of glucosamine was determined at three concentrations (10, 400 and 2000 μg/l). Two series of plasma samples were prepared: in the first case glucosamine was added to blanc plasma before the extraction, as described above, and in the other case glucosamine was added to the sample after protein precipitation to yield the same concentrations. The recovery was calculated by comparing the areas of glucosamine peaks in both cases.

2.5.5. Stability

Short-term stability was determined by analyzing QC samples kept at room temperature for 12 h. Samples were analyzed every 4 h. To investigate long-term stability samples were kept at −20 °C for 10 days. The samples were analyzed after 2, 5 and 10 days of storage. Freeze–thaw stability was determined by analyzing the samples undergoing three freeze (−20 °C)–thaw (room temperature) cycles.

2.6. Application to pharmacokinetic study

The suggested method was applied to determine the plasma concentration of glucosamine in a clinical trial in which 10 healthy male volunteers received a single 1200 mg dose of glucosamine (three pills). Blood samples were collected 0.5, 1, 1.5, 2, 3, 5, 12, 24 and 48 h after oral administration. Samples were centrifuged at 3000 rpm for 10 min, plasma was separated and stored at −20 °C until analyzed. The maximum plasma concentration (C_{max}) and the time of maximum plasma concentration (T_{max}) were determined directly from the concentration–time curve. The elimination rate constant (k_e) was determined as the slope of the linear regression of the log-transformed concentration values versus time. The elimination half-time ($T_{1/2}$) was calculated from the equation $T_{1/2} = \ln 2/k_e$. The area under the concentration–time curve (AUC) from zero to the last data point ($AUC_{0\rightarrow t}$) was obtained according to the linear trapezoidal rule. The AUC from zero to infinity ($AUC_{0\rightarrow\infty}$) was cal-

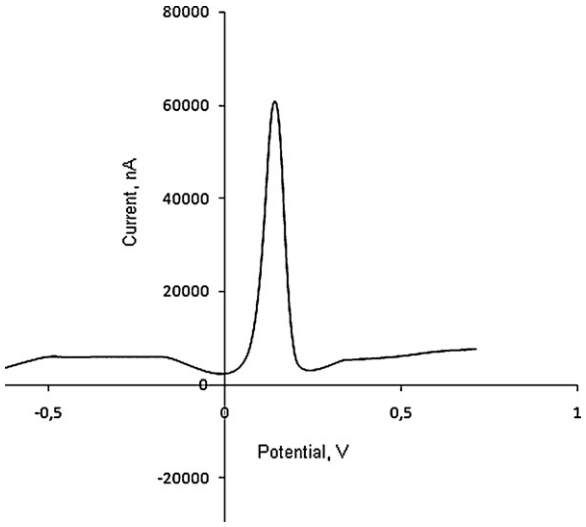

Fig. 2. The current–voltage relationship for the glucosamine in 200 mM sodium hydroxide.

Table 1
The precision and the accuracy of the method.

Concentration added (ng/ml)	Intra-day ($n=5$)			Inter-day ($n=15$)		
	Concentration found (ng/ml) (mean ± S.D.)	Accuracy (%)	Precision (%)	Concentration found (ng/ml) (mean ± S.D.)	Accuracy (%)	Precision (%)
10	9.9 ± 0.3	99.0	3.0	9.6 ± 0.5	96.0	5.2
400	397 ± 7	99.3	1.8	402 ± 11	100.5	2.7
2000	1980 ± 90	99.0	4.5	2090 ± 100	104.5	4.8

culated from the equation $AUC_{0\to\infty} = AUC_{0\to t} + c_t/k_e$, where c_t was the last measured concentration.

3. Results and discussion

3.1. Chromatographic separation and amperometric detection

The suggested conditions allowed rapid separation of glucosamine from interfering endogenous compounds. The retention time of glucosamine was 6.8 ± 0.1 min, and the whole time of the analysis was 12 min. Pulsed voltammetry mode with the quadrupole potential waveform turned to be the most suitable technique of the detection in comparison with fixed-potential amperometric detection.

The first potential of 0.1 V is the detection potential at which the current from glucosamine oxidation is integrated. At the next stage (potential of −2 V) cleaning of the electrode is performed. The potential of 0.6 V is set to maintain an active electrode and at −0.1 V oxide formed at the positive activation potential is reduced. The voltammetric curve for the glucosamine at a high pH value (in 200 mM sodium hydroxide) is shown in Fig. 2.

3.2. Method validation

3.2.1. Selectivity

No interfering peaks from the endogenous plasma compounds were observed at the retention time of glucosamine. The representative chromatograms of blank plasma sample, spiked plasma and a plasma sample, containing glucosamine are shown in Fig. 3.

3.2.2. Linearity

The method showed good linear response ($R^2 = 0.9989$) over the concentration range 10–6000 ng/ml. A typical regression equation was $y = 0.521x + 0.0683$, where y is the area of glucosamine peak, x is the concentration of the glucosamine. The lower limit of quantification (LOQ) and the limit of detection were 10 and 2 ng/ml, respectively.

3.2.3. Precision and accuracy

The data for intra- and inter-day precision and accuracy are presented in Table 1. The results were reproducible and intra- and inter-day precisions were less than 10%. The accuracy values were in the range 96.0–104.5%.

3.2.4. Extraction recovery

The mean extraction recovery for glucosamine at each quality control level (10, 400 and 2000 ng/ml) was 92 ± 8%, 93 ± 6% and 87 ± 8%, correspondently.

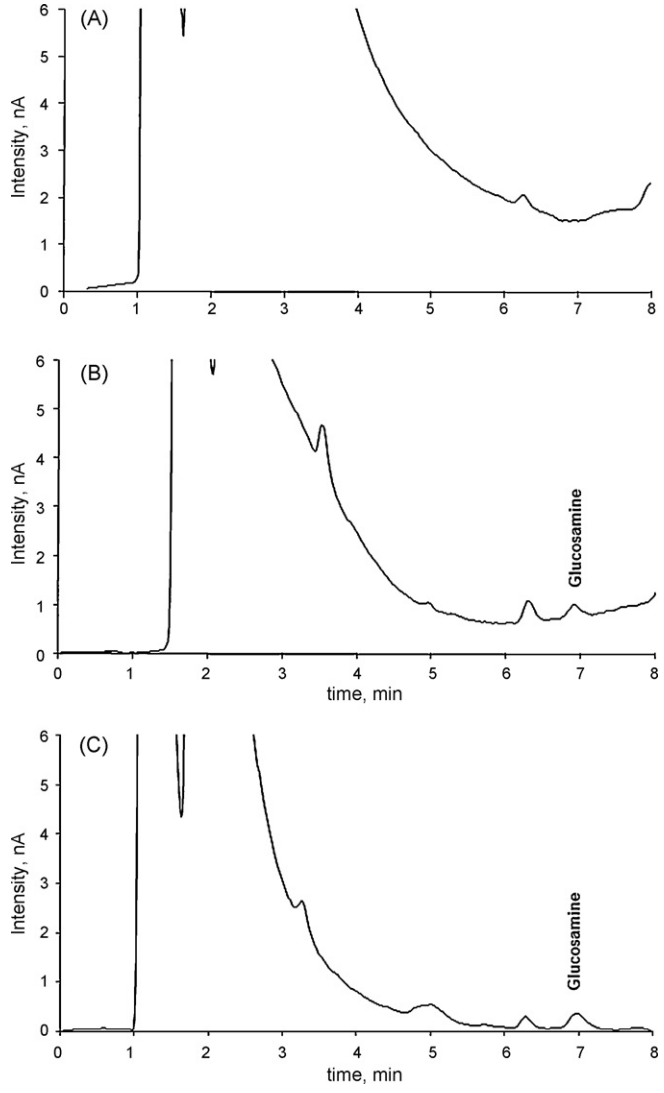

Fig. 3. The chromatogram of (A) blank human plasma, (B) human plasma spiked with glucosamine (concentration of glucosamine 10 ng/ml), (C) a plasma sample obtained at 48 h after a single oral dose (1200 mg) of glucosamine (concentration of glucosamine in sample was determined to be 12 ng/ml).

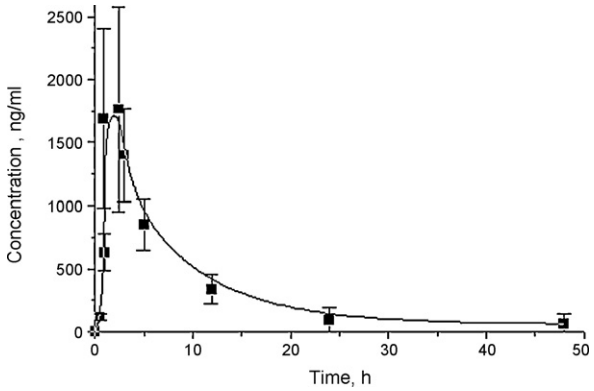

Fig. 4. Mean plasma concentration–time curve of glucosamine after a single oral administration of 1200 mg in 10 healthy male volunteers.

Table 2
The stability of glucosamine in human plasma.

	Accuracy (%) (mean ± S.D.)		
	Concentration added (ng/ml)		
	10	400	2000
Short-term stability (12 h, room temperature)	97 ± 2	96.9 ± 0.8	98.7 ± 0.6
Long-term stability (10 days, −20 °C)	98.5 ± 0.8	99.4 ± 0.7	96.8 ± 0.8
Freeze–thaw stability (three cycles, −20 °C to room temperature)	96 ± 3	98.2 ± 0.5	100 ± 1

3.2.5. Stability

The results demonstrated reliable stability behavior of glucosamine under tested conditions. The data obtained are summarized in Table 2. No significant difference (<5%) was observed between glucosamine concentration at zero time and at the end of the experiments.

3.3. Pharmacokinetic application

The mean plasma concentration–time curve is shown in Fig. 4. Mean maximum plasma concentration of glucosamine was found to be $C_{max} = 1680 \pm 870$ ng/ml at $T_{max} = 1.8 \pm 0.9$ h. The plasma elimination half-time ranged from 4.3 to 7.6 h with a mean value of 5.8 h. $AUC_{0\rightarrow t}$ and $AUC_{0\rightarrow\infty}$ were $14\,390 \pm 8670$ and $14\,910 \pm 8500$ ng h/ml, respectively.

4. Conclusion

The method for the determination of glucosamine in human plasma was developed. In comparison with the reported techniques the suggested one is cheap, simple and may be easily used in clinical laboratories. The method was proved to be specific, sensitive, precise and accurate. It was successfully applied to the determination of the glucosamine plasma concentration after a single oral administration.

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