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Direct injection of human serum and pharmaceutical formulations for glucosamine determination by CE-C^{4}D method

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ABSTRACT

A simple CE-C^{4}D method has been developed for the determination of glucosamine by direct injection of human serum and pharmaceutical samples. Glucosamine was electrokinetically injected and analysed in its protonated form using 20 mM MES/His (pH 6) as background electrolyte in order to separate it from the matrix and to provide a better response to the C^{4}D detector. Separation of glucosamine in human serum and pharmaceutical samples was performed in 3 min without the need for protein precipitation or matrix removal. Good precision in terms of %RSD for the migration time and peak area were less than 1.91% (n = 10). The conductivity signal was linear with glucosamine concentration in the range 0.10–2.50 mg/mL, with a detection limit of 0.03 mg/mL. Recoveries of glucosamine in serum and pharmaceutical samples were 86.5–104.78%. The method was successfully applied for the determination of the glucosamine content in pharmaceutical formulations and validated with high performance liquid chromatography (HPLC). Good agreements were observed between the developed method, label values and the HPLC method. Glucosamine could be detected in spiked serum sample by direct injection. This was not possible by HPLC due to co-eluting interferences.

1. Introduction

Glucosamine (Fig. 1) is an amino sugar occurring naturally in the body, acting as a precursor of glycosaminoglycans which are major components of joint cartilage. Supplemental glucosamine is believed to prevent cartilage degeneration and to treat arthritis [1–3]. Glucosamine is commonly formulated as glucosamine sulfate or glucosamine hydrochloride in combination with other nutraceutical supplements such as chondroitin sulfate and methylsulfonylmethane [4]. In study of the biological role of glucosamine and for quality control in drug production, an accurate and reliable method for analysis of glucosamine in biological fluids and pharmaceutical formulations is required.

Several methods for the analysis of glucosamine have been reported. High performance liquid chromatographic methods (HPLC) have been developed for its determination in pharmaceutical samples [5–8] and biological fluids [9–15]. The relatively polar glucosamine can be separated on normal phase columns such as amino [6,7,14] and cyano columns [12]. However since the compound has no chromophore in its structure, it is therefore hard to detect by measuring the UV absorption of the compound. However the compound can be derivatised to give a strong absorption chromophore [5,16,17]. Many research groups have reported HPLC methods using other detection techniques, such as electrochemical detection [13], refractive index detection [6,18], or MS/MS [12]. Good sensitivity in glucosamine detection could be achieved using fluorometric detection with a derivatization step to convert the glucosamine into a fluorescing compound [5,9]. Capillary electrophoresis (CE) is frequently applied for pharmaceutical analysis as reviewed in intensive reviews [19–22]. For analysis of glucosamine by CE-UV, the determination involves a derivatization step before CE separation (e.g. with anthranilic acid (2-aminobenzoic acid) [23] or o-phthalaldehyde [24]). Conductometric detection, which detects all ionic compounds, is an alternative choice for glucosamine detection. Capacitively coupled contactless conductivity detection (C^{4}D) is often utilized due to its compatibility with the CE system [25–29]. C^{4}D detection can be

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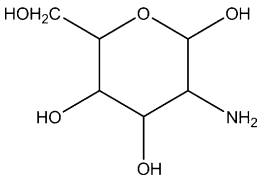

Fig. 1. Chemical structure of glucosamine.

performed on the capillary without the need of any special interface or loss of separation efficiency and resolution. Jáč et al. have reported the use of a high conductivity background electrolyte for the analysis of glucosamine by CE with indirect C^4D detection for pharmaceutical samples [30]. In this work, we present a simple in-house CE method with C^4D detection for the analysis of glucosamine in pharmaceutical samples and spiked human serum. Under the CE conditions, protonated glucosamine (pK_a = 6.91 [31]) was easily separated from neutral matrices which are a major interference in serum sample. The samples could be directly injected and separated within 3 min without the need of any sample preparation except a simple dilution step. The method showed superior performance compared to HPLC method in terms of analysis time, simplicity and less reagent consumption.

2. Materials and methods

2.1. Chemicals, reagents, and samples

Standard glucosamine HCl (purity > 99%) was purchased from Sigma Chemical (USA). L-histidine (His) (purity > 99%) was supplied by Fluka (Japan). 2-(*N*-morpholino) ethanesulfonic acid (MES) was purchased from Acros Organic (USA). Sodium hydroxide was obtained from Merck (Germany). Reverse-osmosis HPLC grade water (RO water) was used for preparation of all solutions.

2.2. Instrumentation and CE-C^4D operation

The separation and analysis of glucosamine was performed in a fused-silica capillary (100/360 μm i.d./o.d., 24 cm effective length, 35 cm total length obtained from Agilent Technologies, Inc. (USA)). Before each analysis, the capillary was conditioned with 0.1 M NaOH for 15 min, water for 10 min and background electrolyte (BGE) for 30 min. Standard solutions of glucosamine and samples were introduced into the CE system by means of electrokinetic injection at 1 kV for 3 s. The high-voltage power supply (Spellman, Model NY 11788, USA) was operated at +7 kV for the CE separation. On capillary detection using C^4D detector was made using two tubular stainless steel electrodes each 2 cm long. The detection gap between the electrodes was 1 mm. The input for the C^4D detector was connected to commercial function generator (Lodestar, Model 2103, Taiwan) to apply ac signal at 100 kHz and 80 V (p/p). The output current was converted to voltage and then rectified and amplified by in-house pre-amplifier. The output dc voltage was monitored by a Powerchrom E-corder (Model 201, Australia). The data recording was analysed using eDAQ PowerChrom software (Australia).

2.3. HPLC system

Separation of glucosamine by HPLC followed the method previously reported by Shao et al. [7]. The HPLC system comprised of a binary pump (Agilent, 1100 series, USA), injector with 20 μL injection loop (Rheodyne, Model 7725, USA) and diode array detector (Agilent, 1100 series, USA). Standard glucosamine and sample were separated on a Waters Spherisorb® amino column (5 μm, 150 mm × 4.6 mm) using a mobile phase of acetonitrile – 20 mM phosphate buffer (50:50, v/v, pH 3.5) at flow rate of 1.5 mL min^{-1} and UV detection at 195 nm.

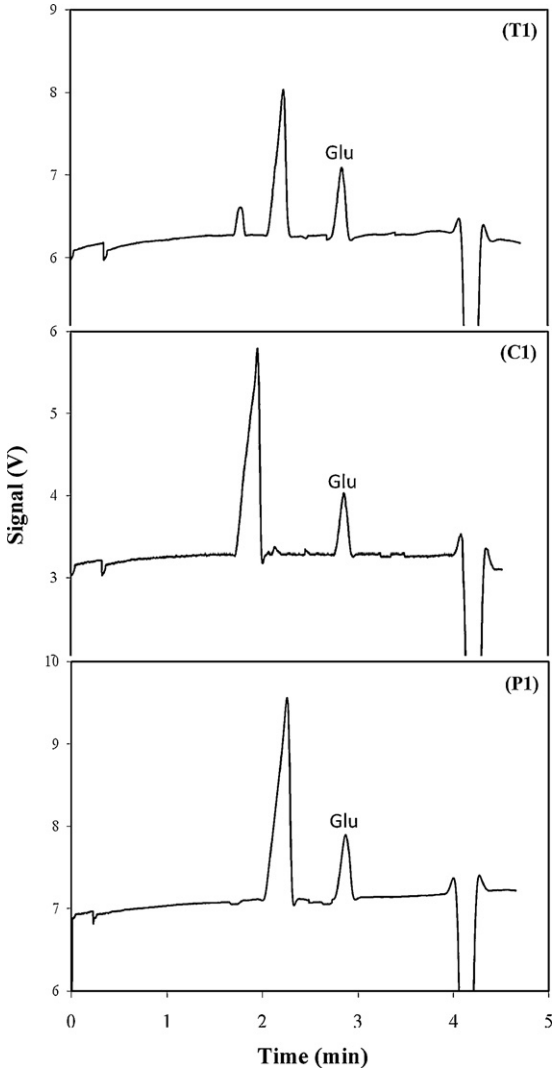

Fig. 2. Selected electropherograms of pharmaceutical samples; powder form (P1), capsule form (C1) and tablet form (T1). CE conditions: 20 mM MES/His buffer pH 6.0, 25 °C, separation voltage +7 kV with electrokinetic injection at +1 kV for 3 s.

2.4. Standard and electrolyte preparation

Stock 10 mg (glucosamine)/mL standard solution was prepared by dissolving 0.6018 g of glucosamine·HCl in RO water and made up to 50.00 mL. The electrolyte solution was prepared from stock solutions of 150 mM MES and 150 mM His. The electrolyte solution was filtered through a 0.45 μm nylon membrane and degassed for 15 min before use.

2.5. Sample preparation

Tablets, capsules and powder form of the drug samples were individually weighed and dissolved in water. The solutions were sonicated for 5 min, centrifuged for 15 min at 1500 rpm before being made up to volume. The sample solutions were diluted and filtered through disposable syringe filter membranes (pore size 0.22 μm) before injection into the CE-C^4D system. Serums obtained from volunteers (n = 10) were stored at −20 °C. Before analysis, the samples were thawed and diluted with water in the ratio of 1:3 (v:v)

Table 1
Intra-day and inter-day precisions for EOF, migration time (t_m) and peak area (PA) of 1.0 mg/mL standard glucosamine (mean ± SD (%RSD)).

	Intra-day	Inter-day
EOF	4.19 ± 0.01 (0.30)	4.20 ± 0.03 (0.77)
t_m (min)	2.88 ± 0.02 (0.80)	2.87 ± 0.03 (1.76)
PA (V s)	5.38 ± 0.20 (1.91)	5.37 ± 0.19 (2.26)

serum:water. Recovery studies were performed at three spiked concentration (0.10, 0.25 and 1.00 mg/mL). Standard glucosamine was spiked into 1.00 mL of the serum solution. The solution was then diluted with water to final volume of 2.00 mL. All the solutions were filtered through filter membranes (pore size 0.22 μm) before CE analysis. The rate of EOF was obtained from the negative peak of water in the sample solution.

3. Results and discussion

3.1. CE separation

In order to achieve separation of glucosamine in a suitable time, various parameters in the CE separation were optimised. The first parameter was the selection of the BGE. The required BGE should have low conductivity giving rise to small joule heating effect. Several BGE's were studied such as His/acetate buffer (pH 4), tris buffer (pH 7), and MES/His (pH 6). The best results in terms of peak shape and sensitivity, were obtained using MES/His buffer (pH 6) as the BGE (data not shown).

Since glucosamine has a pK_a of 6.91 [31], its amino group will be protonated at a pH lower than 7.8. For separating using CE, the analyte must be ionic. The buffer pH was therefore varied between 5 and 7. At pH 5, although the observed EOF was small, the glucosamine was fully protonated resulting in a short separation time. However high background signal was produced at this pH resulting in low sensitivity. At pH 7, the migration time of glucosamine was close to that of EOF. A good separation of glucosamine with good sensitivity was obtained at pH 6 (data not shown). A pH of 6 was therefore selected for the separation condition.

The effect of MES/His concentration on the separation was studied from 2 to 50 mM. Increasing the buffer concentration resulted in narrow peak shape and higher efficiency. However the background signal and noise also increased (data not shown). A 20 mM MES/His solution was found to be the optimum concentration as a compromise between the peak shape and sensitivity.

3.2. Analytical performance of the method

The method gave good linear correlation of the glucosamine concentration versus conductivity signal ($r^2 = 0.9984$) at the concentration range of 0.10–2.50 mg/mL. The linear equation was $y = 0.0051x + 0.0116$, where y is the peak area and x is the glucosamine concentration. Limits of detection and quantification of the method were 0.028 and 0.094 mg/mL, respectively. The LOD was obtained from injection of a glucosamine solution at concentration where the signal (peak height) to baseline noise equal to three (Signal/Noise = 3) while the LOQ was the concentration of glucosamine that produced a signal to baseline noise of ten (Signal/Noise = 10). Precision of the method was examined by repeatedly injecting standard glucosamine within a day (intra-day precision) or between three days (inter-day precision). Good precision was obtained for both the inter- and intra-day experiments as shown by the data in Table 1. A minimum of 20 injections of serum can be performed without loss of separation efficiency or signal.

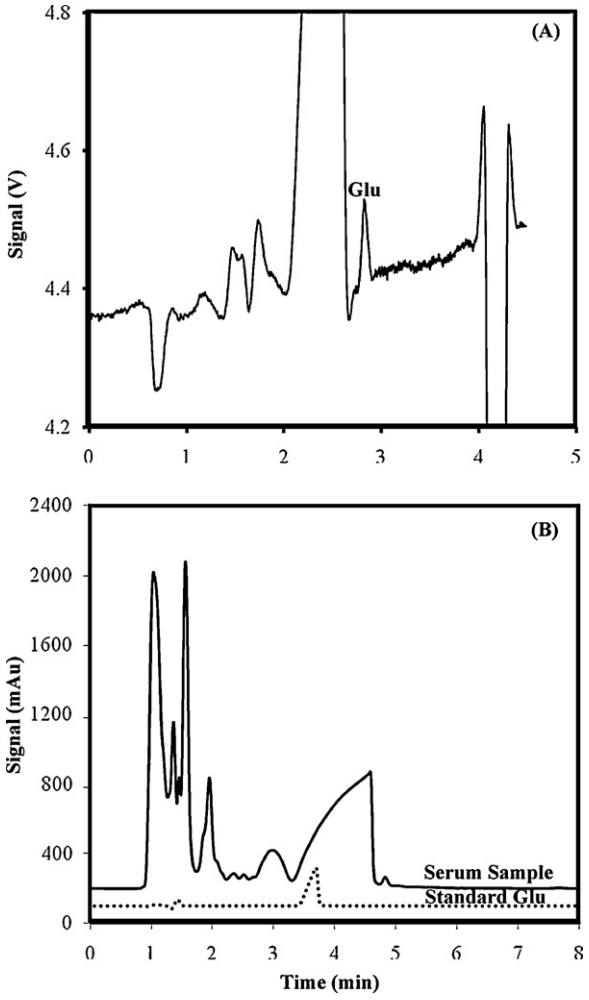

Fig. 3. (A) Electropherogram of spiked human serum (CE conditions as shown in Fig. 2) (B) chromatograms of spiked human serum and standard glucosamine 1.0 mg/mL (HPLC condition; amino column (150 mm × 4.6 mm), mobile phase of acetonitrile – 20 mM phosphate buffer (50:50, v/v, pH 3.5), flow rate of 1.5 ml min^{-1} and UV detection at 195 nm).

3.3. Applications to pharmaceutical samples and human serum

3.3.1. Pharmaceutical samples

The method was applied to determine the glucosamine content in various types of drug samples formulated in tablet, capsule, and powder forms. The results agreed well with label values and results from the HPLC method with no significant difference at 95% confidence (Fig. 2 and Table 2). Good recoveries were obtained between 86.5 and 104.7%.

3.3.2. Human serum sample

Human serum (diluted 3:1 fold) was directly injected into the developed system in order to examine the potential of the method for the direct analysis of glucosamine in serum samples which normally require sample preparation steps such as protein precipitation or matrix removal before analysis. For our method, the spiked serum sample was only filtered before being directly injected into the CE-C^4D system by mean of electrokinetic injection. Good separation of glucosamine from the matrix interferences was obtained within 3 min (Fig. 3A) while separation of glucosamine in the serum by direct injection could not be achieved by the HPLC method due to interference from the matrix (Fig. 3B). This CE method has advantages over the HPLC method because the electrokinetic injection is partially selective towards cations, resulting in less interference. In

Table 2
Quantification of pharmaceutical formulations and recovery study by the developed CE-C^{4}D and HPLC methods. Glucosamine drug samples: tablet form (T1), capsule form (C1–C4) and powder form (P1–P4).

Samples	CE-C^{4}D		HPLC	Values on the label
	%Recovery ± SD	Quantification (mg/sample) ± SD	Quantification (mg/sample) ± SD	
T1	99.3 ± 5.9	1.44 ± 0.01	1.40 ± 0.00	1.50[a]
C1	87.4 ± 5.2	0.54 ± 0.01	0.56 ± 0.01	0.50[b]
C2	89.0 ± 5.5	0.53 ± 0.02	0.56 ± 0.01	0.50[b]
C3	87.6 ± 2.8	0.27 ± 0.00	0.27 ± 0.00	0.25[b]
P1	104.7 ± 3.0	1.55 ± 0.02	1.63 ± 0.03	1.50[c]
C4	86.5 ± 3.3	1.08 ± 0.02	0.95 ± 0.00	1.00[b]
P2	101.3 ± 7.2	1.53 ± 0.06	1.55 ± 0.00	1.50[c]
P4	102.8 ± 5.1	1.53 ± 0.04	1.63 ± 0.01	1.50[c]
P3	102.4 ± 0.7	1.52 ± 0.03	1.67 ± 0.01	1.50[c]

$n = 3$, n is number of injection.
[a] mg/tablet.
[b] mg/capsule.
[c] mg/sachet.

addition, with the CE method, after the analytes exit the separation capillary, all the remaining compounds can be easily washed from the capillary resulting in shorter analysis times compared to HPLC. Recoveries at three different spiked concentrations of 0.10, 0.25, and 1.00 mg/mL for human serum were 95.1–100.2%.

4. Conclusion

In this rationale we have developed a direct CE-C^{4}D method for the determination of glucosamine in pharmaceutical formulations and in human serum. The method provided good precision, rapidity and simplicity compared with the HPLC technique and it was successfully applied to the quantitfication of glucosamine in pharmaceutical drugs. The results were not significantly different to that of the HPLC method and values as shown on the lable. The method was also shown to have advantages for human serum samples in terms of simplicity and rapidity since the sample could be directly injected into the CE-C^{4}D system without the need for a sample preparation step.

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