ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
20 (1999) 807–814

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Determination of the nutraceutical, glucosamine hydrochloride, in raw materials, dosage forms and plasma using pre-column derivatization with ultraviolet HPLC

Zhongming Liang, James Leslie, Abimbola Adebowale, Mohammed Ashraf, Natalie D. Eddington *

Pharmacokinetics-Biopharmaceutics Laboratory, Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, 100 Penn Street, AHB 540C, Baltimore, MD 21201, USA

Received 30 November 1998; received in revised form 12 April 1999; accepted 5 May 1999

Abstract

A selective and specific high performance liquid chromatography method was developed to quantitate glucosamine hydrochloride in raw materials, dosage forms and plasma. Reverse phase chromatography using pre-column derivatization with phenylisothiocyanate, and ultraviolet detection ($\lambda = 254$ nm) was used to quantify the eluate. The mobile phase consisted of MeOH/H_2O/CH_3COOH (10:89.6:0.04) and was pumped at a flow rate of 1.2 ml/min. The standard curves for glucosamine hydrochloride showed linearity ($r \geq 0.99$) over the selected concentration range from 6.65 to 16.63 μg/ml for raw materials and dosage forms. The precision of the dosage form assay, expressed as the % relative standard deviation (R.S.D.), was $< 5\%$ at all concentrations. The intra-day and inter-day accuracy, as indicated by the relative error (R.E.), ranged from -2.54 to 2.70% for glucosamine hydrochloride. For the plasma assay, beagle dog plasma was used to prepare standard curves in the concentration range of 1.25–20 μg/ml. Precipitation of plasma proteins was accomplished with acetonitrile to separate interfering endogenous products from the compound of interest. The supernatant was derivatized using phenylisocyanate in phosphate buffer (pH = 8.3) and subsequently evaporated to dryness under a nitrogen stream at 42°C. The residue was dissolved in 250 μl mobile phase and injected onto the chromatographic system. The assay was linear in concentration ranges of 1.25–20 μg/ml ($r \geq 0.999$). Intra- and inter-day precision was ≤ 5.23 and 5.65%, respectively and the intra- and inter-day accuracy, indicated by R.E., ranged from -8.6 to 10.35%. The method was found to be specific and with excellent linearity, accuracy and precision and is well suited for the quantitation of glucosamine hydrochloride in raw materials, dosage forms, and pharmacokinetic studies.

Keywords: Glucosamine hydrochloride; Nutraceutical; Phenylisothiocyanate; Arthritis

* Corresponding author. Tel.: +1-410-706-6710; fax: +1-410-706-6580.
E-mail address: eddingto@pharmacy.ab.umd.edu (N.D. Eddington)

0731-7085/99/$ - see front matter
PII: S0731-7085(99)00085-0

1. Introduction

Glucosamine hydrochloride is currently used for the treatment of degenerative joint disease in small animals and represents a novel approach for the treatment of degenerative conditions. Glucosamine, an amino monosaccharide, is a natural component of glycoproteins found in connective tissues and gastrointestinal mucosal membranes. It is a precursor of the disaccharide unit of glycosaminoglycans (GAGs) which are the building-blocks of the articular cartilage, the proteoglycans [1–5]. Numerous reports have recently focused on the utility of glucosamine for the treatment of osteoarthritis in dogs [6,7] and horses [8,9].

Glucosamine is considered a dietary supplement by the Food and Drug Administration and as such the content uniformity as well as the bioavailability of glucosamine products are rarely determined. There is a need to accurately determine the content of products containing glucosamine, especially since numerous products are marketed for the treatment of osteoarthritis. Previous analytical methods to determine glucosamine bioavailability have relied on radiolabeling the compound [10,11]. Radioactivity has been used to quantitate the concentration of glucosamine and metabolites in pharmacokinetic studies as well as for determining content in glucosamine products. Using radioactivity to quantitate glucosamine may potentially confound results since the labeled compound and any degradation products will be quantified. Unfortunately, this precludes determining the stability of glucosamine products under various experimental conditions or using radioactivity to accurately determine bioavailability.

Other methods to quantify glucosamine have been reported for compositional analysis of amino sugars following pre-column derivatization as well as quantifying glucosamine in certain bacterial cell walls [12–14]. The purpose of this work was to develop a precise and accurate HPLC method for glucosamine that could be routinely applied to the quantitation of this agent in raw materials and to evaluate the content uniformity of products containing glucosamine. In addition, to determine the bioavailability properties of glucosamine hydrochloride, the second objective was to develop and validate a selective, specific and reproducible HPLC method for the quantitation of intact glucosamine hydrochloride in plasma.

2. Experimental

2.1. Materials and reagents

D(+) Glucosamine (2-amino-2-deoxy-D-glucose) hydrochloride (Fig. 1) was purchased from Sigma (St Louis, MO). Cosequin®/Cosamin®, Cosequin DS®/Cosamin DS® was donated by Nutramax Laboratories (Baltimore, MD). The capsule products contained 250 or 500 mg (DS) of glucosamine hydrochloride, and chondroitin sulfate and manganese ascorbate. Raw materials of glucosamine were purchased or distributed by Zhejiang (lot # 961013, lot # 961014, China) and Marcor Development (lot # MD-G60426, and lot I-20; China). Methanol, acetonitrile, phenylisothiocyanate (PITC), sodium phosphate and acetic acid were purchased from J.T. Baker (Phillipsburg, NJ). All chemicals and solvents were ACS analytical grade or HPLC grade. Deionized water was prepared by an ultrapure water system Pyrosystem Plus® (Hydro, Research Triangle Park, NC).

2.2. Standard and sample preparation for raw materials and dosage form

Glucosamine hydrochloride standards were prepared in water in the concentration range from 6.65 to 16.6 mg/ml. A stock solution was prepared at a concentration of 1.0 mg/ml and serial dilutions were made to give concentration of standards of 6.65, 10.7, 12.0, 13.3, 14.6, and 16.6

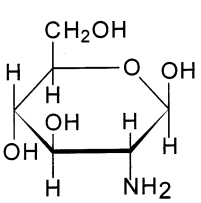

Fig. 1. Chemical structure of glucosamine.

μg/ml. To each standard, 250 μl of a 0.3 M phosphate buffer (pH 8.0) and 250 μl of 5% PITC were added. Samples were vortexed, allowed to react at room temperature for 10 min and were evaporated to dryness at about 50°C under nitrogen. After the derivatization was complete, 200 μl of mobile phase was added and 20 μl was injected onto the equilibrated HPLC system.

2.3. Preparation of standards and plasma extraction procedure

Standards were prepared in concentrations ranging from 1.24 to 20 μg/ml in beagle dog plasma from a stock solution of glucosamine hydrochloride (100 μg/ml). The internal standard, galactosamine, was prepared at a concentration of 200 μg/ml. Twenty microliters of the internal standard (galactosamine) was added to 0.40 ml plasma. Samples were vortexed for 10 s and 500 μl of acetonitrile was added. The samples were again vortexed and centrifuged ($3000 \times g$) for 10 min. The resulting supernatant was transferred to a 3.0-ml disposable glass tube and 250 μl of 0.3 M Na_2HPO_4 (pH 8.3) and 250 μl of 5% PITC were added. Samples were vortexed, allowed to react at room temperature for 10 min and were evaporated to dryness at 50°C under nitrogen. After the derivatization was complete, 200 μl of mobile phase was added and 20 μl was injected onto the equilibrated HPLC system.

2.4. Instrumentation

The samples were analyzed by an HPLC system consisting of two Waters 6000A solvent delivery systems, a Waters WISP 710B automatic injector, and a Waters 486 Ultraviolet Detector ($\lambda = 254$ nm) (Waters Associates, Milford, MA). The detector was coupled with an HP 3384 Integrator plotter (Hewlett-Packard, Avondale, PA) and a C_{18} analytical column (Bondclone (30 cm × 3.9 mm i.d., 5 μm, Phenomenex, Macclesfield Cheshire, UK) was used for separation. The buffer component of the derivatized solution was prepared with deionized water and the pH was adjusted to 8.0 using concentrated phosphoric acid. The mobile phase consisted of $MeOH/H_2O/CH_3COOH$ (10:89.96:0.04) and was filtered through a 0.45-μm nylon filter and degassed under ultrasound and vacuum for 15 min. The mobile phase was delivered at a flow rate of 1.2 ml/min and the eluent was evaluated at a wavelength of 254 nm.

2.5. Assay validation

2.5.1. Linearity and range

Linearity of the dosage form assay was determined by analysis of three replicates of six concentrations (6.65, 10.65, 11.98, 13.31, 14.64 and 16.63 μg/ml) by least squares regression. The linear detector response for the plasma assay was tested as follows. Three determinations ($n = 3$) from a minimum of five concentration levels (1.25, 2.5, 5.0, 10.0 and 20 μg/ml) of the analyte concentration were evaluated by least-squares regression. A weight of $1/y$ was used to determine slopes, intercepts and correlation coefficients. The minimum acceptable coefficient to establish linearity was set at 0.95 a priori.

2.5.2. Method precision and percent recovery

Precision of the assay was established by analysis of three replicates ($n = 3$) of a standard solution of the analyte at the following concentrations: 6.65, 10.65, 11.98, 13.31, 14.64, 16.63 μg/ml for the dosage form assay and 1.25, 2.5, 5.0, 10.0, 20 μg/ml for the plasma assay. To determine intra-day precision of the assay, replicate ($n = 3$) blood samples of five different concentrations were analyzed. To determine inter-day precision, replicate blood samples ($n = 3$) were analyzed on three different days. The percent relative standard deviations of the assay results were determined.

Extraction efficiency was determined by comparing replicate ($n = 9$) peak height ratios of extracted plasma samples vs. unextracted water standards for the 2.5, 5, 10 and 20 μg/ml concentrations. The percent recovery was determined by the following equation:

$$\% \text{ Recovery} = \frac{\text{Peak height ratio (plasma standard)}}{\text{Peak height ratio (water standard)}}$$

2.5.3. Accuracy

Accuracy of the method was determined by calculating the mean concentration of three replicate ($n = 3$) solutions at concentrations ranging from 6.65 to 16.63 μg/ml for the dosage form method and 1.25–20 μg/ml for plasma. Method standards in the concentration range of 2.5–250 μg/ml from three different runs performed over several days were used to check for accuracy. The means of the three runs were calculated and compared to the spiked value to determine the percentage difference between the mean and the spiked value (amount added). The percentage relative error was determined as:

$$\%\ \text{Relative error (R.E.)} = \frac{[\text{mean} - \text{spiked}]}{[\text{spiked}]} \times 100$$

Between and within run accuracy were also determined.

2.5.4. Specificity, stability and robustness

Specificity was evaluated by testing samples to ensure that no interference from components of the mobile phase, degradation products, excipients or beagle dog plasma were obtained in the presence of the analyte of interest. Stability of the analyte in the dosage form standards and beagle dog plasma at room temperature and at −85°C as well as two freeze thaw cycles were determined. This was performed by analyzing the standard solutions in triplicate at different times after storage. Robustness was assessed by determining the retention times of glucosamine hydrochloride using two different lots of the same analytical column and two different batches of mobile phase.

2.6. Analysis of raw materials

Various lots and suppliers of glucosamine hydrochloride raw materials were evaluated. One hundred milligrams of the glucosamine hydrochloride raw material samples were weighed, placed in a 100-ml volumetric flask and an appropriate volume of water was added. The solution was sonicated for 20 min and an aliquot of this solution was transferred to a test tube and diluted to a suitable concentration within the standard concentration range. To this aliquot 250 μl of phosphate buffer (0.3 M, pH 8.0) and 5% PITC were added. The analysis was continued as described above for the derivatization of glucosamine

2.7. Analysis of capsules

Twenty capsules containing 250 mg of glucosamine each were weighed. The contents of each capsule were transferred quantitatively to a 250-ml volumetric flask and about 150 ml of water added. The mixture was sonicated for 20 min and brought to volume with water. A portion of each solution was centrifuged ($3000 \times g$ for 5 min) and 20 μl derivatized with PITC. Simultaneously, a standard solution of glucosamine hydrochloride was treated in a similar manner. The resulting solutions were chromatographed in duplicate.

2.8. Pharmacokinetic study

A study was designed to investigate the pharmacokinetics of glucosamine hydrochloride in beagle dogs. The protocol was approved by the Animal Research Committee at the University of Maryland, School of Pharmacy. A quantity of 2000 mg of glucosamine hydrochloride (Cosequin DS/Cosamin DS, four capsules) was orally administered to a male dog (15 kg). Serial blood samples (2.0 ml) were collected via the jugular vein over a 24-h period into EDTA tubes. Plasma samples were separated by centrifugation ($3000 \times g$) for 10 min and immediately frozen at −85°C.

3. Results

3.1. Resolution

Fig. 2a–c represents chromatograms of extracted blank, calibration standard containing 6.65 μg/ml of glucosamine hydrochloride and a raw material sample containing 6.25 μg/ml of glucosamine hydrochloride, respectively. The assay was found to be specific for glucosamine hydrochloride and no interfering peaks from

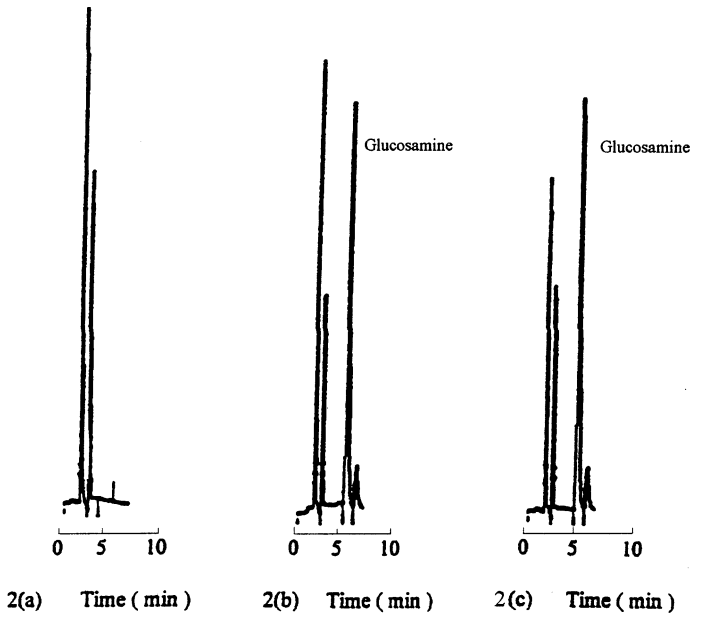

Fig. 2. Chromatograms of (a) extracted blank sample, (b) glucosamine standard, and (c) glucosamine sample (raw material).

chondroitin, manganese ascorbate or excipients were found. Fig. 3a and Fig. 3b represent chromatograms of extracted blank beagle dog plasma and a calibration standard containing 5 μg/ml of glucosamine hydrochloride, respectively. The assay was found to be specific for glucosamine hydrochloride and no interfering peaks from degradation products, EDTA, internal standard or plasma were detected. Moreover, the peaks of glucosamine hydrochloride and the internal standard were sufficiently separated with typical retention times of 14.4 min for glucosamine hydrochloride and 12.2 min for the internal standard.

3.2. Validation assay precision

The concentrations of the standards were calculated using the observed peak areas and the straight line parameters and all were within 5% of the nominal value. These observations and the correlation coefficients of greater than 0.999 showed a satisfactory fit to the linear model. The standard curves for glucosamine hydrochloride showed linearity over the selected concentration range from 6.65 to 16.63 μg/ml for raw material and dosage form with consistent slopes and excellent correlation coefficients ($r \geq 0.99$) throughout the validation runs. The dosage form intra-day and inter-day precision data for glucosamine hydrochloride are listed in Table 1. The intra-day and inter-day % R.S.D. was 4.18% or less for all standards. The precision of the assay, expressed as the % R.S.D., was $< 5\%$ at all concentrations. Table 2 summarizes the dosage form intra-day and inter-day accuracy data for glucosamine hydrochloride. The intra-day and inter-day accuracy, as indicated by R.E., ranged from -2.54 to 2.70% for glucosamine hydrochloride.

The standard curves for glucosamine hydrochloride showed linearity over the selected concentration range from 1.25 to 20 μg/ml in plasma with consistent slopes and excellent correlation coefficients ($r \geq 0.99$) throughout the validation runs. The intra-day and inter-day precision data for glucosamine hydrochloride in beagle dog plasma are listed in Table 3. The intra-day and inter-day % R.S.D. ranged from 0.65 to 5.26% and 1.28 to 5.64%, respectively. Table 4 summarizes the intra-day and inter-day accuracy data for

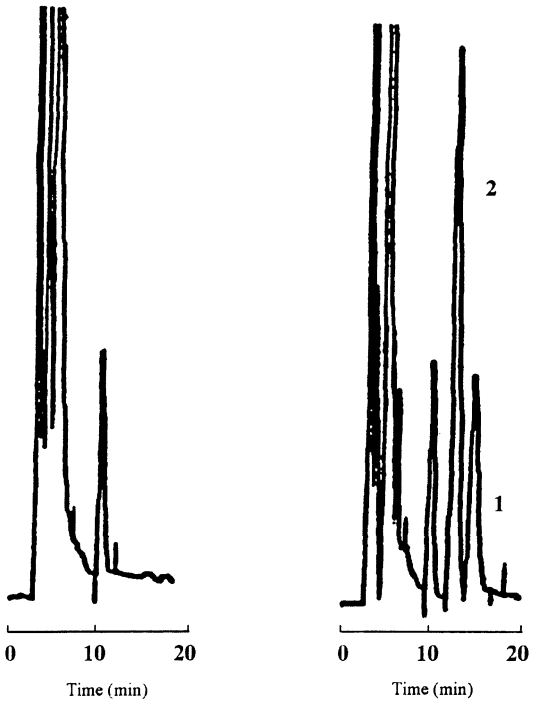

Fig. 3. Typical chromatograms of glucosamine hydrochloride in plasma: (A) extracted blank plasma; (B) glucosamine hydrochloride standard, 13.3 μg/ml. Peaks 1 and 2 represent glucosamine hydrochloride and galactosamine, respectively.

glucosamine hydrochloride in beagle dog plasma. The intra-day and inter-day accuracy, as indicated by R.E., ranged from −1.52 to 7.44% and −8.6 to 10.35%, respectively. After the comparison of the PARs for extracted plasma standards and water standards the extraction recovery was found to be 98.68, 100.0, 99.92, 104.12 for the 2.5, 5, 10 and 20 μg/ml standards, respectively.

Table 1
Intra-day and inter-day assay precision for the dosage form assay of glucosamine hydrochloride

Concentration (μg/ml)	Intra-day (% R.S.D.)	Inter-day (% R.S.D.)
16.63	2.93	0.99
14.64	0.47	2.04
13.31	4.18	3.01
11.98	0.51	3.18
10.65	1.27	1.71
6.65	1.45	1.38

Table 2
Intra-day and inter-day assay accuracy for the dosage form assay of glucosamine hydrochloride

Concentration (μg/ml)	Intra-day (% R.E.)	Inter-day (% R.E.)
16.63	1.65	2.22
14.64	−2.54	−0.75
13.31	−1.25	−1.95
11.98	0.90	−2.17
10.65	1.16	0.85
6.65	0.92	2.70

3.3. Stability

Stability of glucosamine hydrochloride was evaluated by comparing the mean peak area of the 13.31 μg/ml dosage form standard which had been in storage for 10 days at room temperature with the peak area of a freshly prepared standard. The mean peak area of the stored standard was within 99% of the freshly prepared standard. Stability of glucosamine hydrochloride in plasma was evaluated by comparing the mean peak area of the 2.5, 5.0 and 10 μg/ml standard and freshly prepare standard under identical assay conditions. The glucosamine hydrochloride was stable after 24 h when stored at room temperature (25°C), 3 months storage at −85°C and through two freeze thaw cycles from −85°C. The variation due to using different analytical columns and mobile phases was found to be negligible under the experimental chromatographic conditions.

Table 3
Intra-day and inter-day assay precision for glucosamine hydrochloride in beagle dog plasma

Concentration (μg/ml)	Intra-day (% R.E.)	Inter-day (% R.E.)
1.25	2.19	2.90
2.50	5.26	5.64
5.00	3.96	3.25
10.0	2.13	2.20
20.0	0.65	1.28

Table 4
Intra-day and inter-day assay accuracy for glucosamine hydrochloride in beagle dog plasma

Concentration (μg/ml)	Intra-day (% R.E.)	Inter-day (% R.E.)
1.25	4.88	−8.6
2.50	1.10	10.35
5.00	7.44	−1.04
10.0	0.08	0.40
20.0	−1.52	−0.40

3.4. Raw material, dosage forms and pharmacokinetic evaluation

The utility of the derivatized HPLC method for glucosamine was assessed by the quantitation of glucosamine content in raw materials and dosage forms. Table 5 summarizes the content of glucosamine in various batches of raw materials and Table 6 presents the content uniformity of Cosequin capsules using the validated method. A plasma concentration vs. time profile following an oral dose of glucosamine hydrochloride (2000 mg) to a representative animal is shown in Fig. 4. This profile highlights the sensitivity of the assay as well as its utility since it is possible to follow the time course of glucosamine after a single oral dose.

4. Discussion

Glucosamine hydrochloride does not have a chromophore absorbing in the wavelength range useful for liquid chromatography with ultraviolet detection. Therefore it was decided to make a derivative of glucosamine hydrochloride that could be detected by ultraviolet absorption. Based on the derivatization of amino acids and sugars with PITC [12–15], we describe the derivatization of glucosamine hydrochloride in dosage forms and plasma. A high performance liquid chromatographic method was developed and validated for the analysis of glucosamine hydrochloride powder, capsules and in plasma. The method involved derivatization of glucosamine by PITC followed by chromatography on an octadecyl (C_{18}) column

Table 5
Content of glucosamine hydrochloride as % w/w of the label amount in various brands of raw materials[a]

Product	Raw material
Zhejiang, lot # 961013 (China)	99.77 (2.8)
Zhejiang, lot # 961014 (China)	101.96 (1.74)
Marcor Development Corp., lot # MD-60426	99.13 (3.92)
Lot I-20 (China)	99.95 (3.35)

[a] Values are mean with S.D. in parentheses.

Table 6
Analysis of glucosamine hydrochloride in capsules (Cosequin®—9602B006, Drum # 2)

Capsule	Weight	Assay (mg)	Percent of nominal
1	649.1	243	97.2
2	649.0	255	102
3	666.6	259	103.6
4	644.1	259	103.6
5	657.5	256	102.4
6	669.1	246	98.4
7	647.1	233	93.2
8	644.1	240	96.0
9	641.2	243	97.2
10	657.9	244	97.6
Mean		247.8	
S.D.		8.9	
R.S.D. %		3.6	

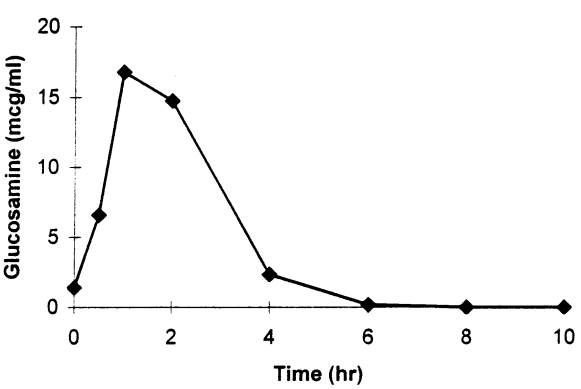

Fig. 4. Plasma concentration vs. time profile from a representative beagle dog following an oral dose of Cosequin DS®/Cosamin DS® (glucosamine hydrochloride, 2000 mg).

with ultraviolet detection at 254 nm. Glucosamine hydrochloride, under the optimized conditions, was eluted from the reverse phase column at a retention time of 14 min, as shown in Fig. 2. Blank solution treated with PITC showed no peaks at the retention time of the glucosamine derivative. Linearity, precision, specificity and stability of glucosamine and the PITC derivative were tested and found to be satisfactory for the analysis of glucosamine hydrochloride.

In initial experiments, galactosamine hydrochloride was tested as a possible internal standard. There was good resolution of the derivatives of glucosamine and galactosamine, but in subsequent experiments it was found that there was no advantage in using an internal standard for the dosage form analysis. Excipients that are likely to be present in the capsule were tested and found not to interfere chromatographically. It is concluded that the method described above is suitable for the analysis of glucosamine in dosage forms.

The assay was found to be specific for glucosamine hydrochloride with no interfering peaks from beagle dog plasma. The standard curves displayed linearity over the selected concentration range (1.25–20 μg/ml). The precision and accuracy data showed that the assay method is consistent and reliable with low values of percent relative error. No degradation products of glucosamine hydrochloride were detected after 24 h at room temperature or after 3 months storage at −85°C. The method was found to be robust with respect to deliberate variations in method parameters. The method can be used to quantitate glucosamine hydrochloride in plasma obtained from pharmacokinetic studies with beagle dogs.

5. Conclusions

In summary, a specific, precise, accurate and rugged assay for the quantitation of glucosamine hydrochloride in dosage forms and plasma has been developed and validated. The accuracy and precision data show that this method is consistent and reliable with low values of relative error and R.S.D. for standards over the concentration range examined. This chromatographic method offers a direct quantitation of intact glucosamine hydrochloride with the use of pre-column derivatization with phenylisothiocyanate. This assay can be used for content uniformity testing of dosage forms containing glucosamine hydrochloride, quality control of raw materials and bioavailability studies.

Acknowledgements

This work was supported by a grant from Nutramax Laboratories, Incorporated.

References

[1] H.W. Greiling, W. Stuhlsatz, U. Tillmans, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, 1984, pp. 60–69.
[2] A. Conte, N. Volpi, L. Palimiera, I. Bahous, G. Ronca, Arzneim.-Forsch. Drug Res. 45 (1995) 918–925.
[3] M.L. Huber, R.L. Bill, Compend. Contin. Educ. Pract. Vet. 16 (1994) 501–504.
[4] R.J. Todhunter, G. Lust, J. Am. Vet. Med. Assoc. 204 (1994) 1245–1251.
[5] R.D. Altman, D.D. Dean, O.E. Muniz, Arthritis Rheum. 32 (1989) 1300–1306.
[6] P.S. McNamara, S. Barr, H.N. Erb, Am. J. Vet. Res. 57 (1996) 1390–1394.
[7] P.S. McNamara, S.A. Johnston, R.J. Todhunter, Vet. Clin. North Am. Small Anim. Pract. 27 (1977) 863–867.
[8] R.R. Hanson, L.R. Smalley, G.K. Huff, S. White, T.A. Hammad, Equine Pract. 19 (1997) 9–11.
[9] R.R. Hanson, Equine Pract. 18 (1996) 18–22.
[10] I. Setnikar, C. Glachetti, G. Zanoio, Pharmatherapeutica 3 (1984) 538.
[11] R. Setnikar, S. Palumbo, G. Canali, G. Zanolo, Arzneim.-Forsch. Drug. Res. 43 (1993) 1109–1113.
[12] J. Diaz, J.L. Lliberia, L. Comellas, F. BrotoPuig, J. Chromatogr. 719 (1996) 171–179.
[13] S.R. Hagen, J. Chromatogr. 632 (1993) 63–68.
[14] K.R. Anumula, P.B. Taylor, Anal. Biochem. 197 (1991) 113–120.
[15] B.A. Bindlingmeyer, S.A. Cohen, T.L. Tarvin, J. Chromatogr. 336 (1984) 93–104.