GLUCOSAMINE BINDING TO PROTEINS IN PLASMA AND SYNOVIAL FLUID AND BLOOD CELL/PLASMA PARTITIONING IN MOUSE AND MAN *IN VITRO*

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SUMMARY

Protein binding of [^{14}C]glucosamine (400, 1000 and 4000 ng/ml) was evaluated in human and mouse plasma and in human synovial fluid. Blood cell/plasma partitioning in human and mouse was also determined. There was no measurable protein binding of [^{14}C]glucosamine. Its association with human and mouse blood cells ranged from 43-47% and from 27-29%, respectively. Therefore, the unbound (pharmacologically active) fraction of glucosamine in plasma and at the site of action (the joint) is the same. Protein binding displacement drug-drug interactions are unlikely during the clinical use of crystalline glucosamine sulfate. No corrections are needed, either for unbound fraction when comparing human and mouse pharmacokinetic data or for blood cell/plasma partitioning to assess glucosamine total blood clearance from plasma data in these two species.

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KEY WORDS

crystalline glucosamine sulphate, plasma protein binding, blood cell/plasma partitioning, drug-drug interactions, synovial fluid

INTRODUCTION

Osteoarthritis (OA), previously called degenerative joint disease, is most often characterized by the insidious progressive destruction of articular cartilage and concomitant changes to other articular structures, such as bones, synovium, joint capsules, ligaments, menisci, bursae and peri-articular muscles. It is a complex process which involves biomechanics, accelerated and inadequate tissue repair, and production of inflammatory mediators including degradative enzymes. The standard classification divides OA into those forms for which the cause is known (secondary OA) and those for which the cause is unknown (primary or idiopathic) /1/.

Several clinical studies have indicated that crystalline glucosamine sulfate (CGS) is effective in controlling knee OA symptoms and disease progression /2-4/. In particular, two randomised, placebo-controlled, double-blind trials of 3 years duration in patients with knee OA showed that this symptom-modifying effect is sustained over long-term treatment courses /2,3/. Moreover, both studies indicated that the drug also has a structure-modifying effect, as assessed by measurement of joint space narrowing using validated techniques on standardized plain radiographs /4-8/. Another, recently completed, trial (the GUIDE study) confirmed the symptomatic results described above and indicated that, at a dose of 1500 mg/day for six months, crystalline glucosamine sulfate provided an analgesic effect that was significantly superior to that observed after administration of placebo /9/. In the same study and depending on the selected outcome measures, the effect observed after the administration of the currently preferred symptomatic medication in OA (acetaminophen), was not always different from that observed after the administration of placebo /9/.

Knee OA is a chronic disease that affects mainly elderly individuals who are often being treated for other concurrent conditions. CGS is marketed in Europe as a prescription drug and thus it is important to evaluate the potential for drug-drug interactions produced

by CGS to provide this information to prescribing physicians, to ensure that the safety and effectiveness of CGS and of the other medications administered to patients taking CGS are maintained. Drug-drug interactions originating from plasma protein binding displacement and consequent increase in the unbound fraction with alteration in the safety and/or efficacy profile of the displaced drug are uncommon but when they occur might generate safety and/or efficacy concerns /10-12/. It is therefore important to asses the plasma protein binding of crystalline glucosamine sulfate to be able to predict displacement drug-drug interactions. In addition, the site of action of glucosamine is the knee, and the synovial fluid in the joint was found to contain significant concentrations of glucosamine when this biological fluid was collected from patients with OA receiving crystalline glucosamine sulfate orally at the therapeutic dose /13,14/. In order to compare the concentration achieved in plasma and synovial fluid after oral administration of crystalline glucosamine sulfate, it is highly relevant to know whether or not the unbound (and pharmacologically active) fraction is the same in the two compartments to allow a direct comparison of the systemic exposure with that at the site of action. Notwithstanding the importance of plasma and synovial protein binding, information on these parameters is scant /15,16/ due to the fact that the pharmacologically and clinically relevant glucosamine concentrations in plasma and synovial fluid after therapeutic doses of crystalline glucosamine sulfate became available only recently /13,14/.

Since several preclinical pharmacology OA models have been developed in the mouse, it is also relevant to assess the plasma protein binding of glucosamine in this species to assess its relevance (as far as the free fraction is concerned) to test the pharmacological effects of crystalline glucosamine sulfate in these OA models. Finally, pharmacokinetic studies in humans and animals need to take into consideration blood cell/plasma partitioning in order to provide a more accurate estimation of the total clearance from plasma data and to further assess the relevance of the animal species in which to investigate the pharmacokinetics, toxicity, and pharmacodynamics of crystalline glucosamine sulfate.

The aim of the present investigation was to assess *in vitro* the binding of crystalline glucosamine sulfate to human and mouse plasma proteins and to human synovial fluid proteins and to assess the

blood cell/plasma partitioning of glucosamine in human and mouse blood.

MATERIALS AND METHODS

Ethanol (purity 100%; AnalR grade), dipotassium orthophosphate (purity 100%; AnalR grade), and acetonitrile (purity 100%, HiPerSolv grade) were obtained from VWR/Prolabo, Lutterworth, UK.

Sodium dihydrogen orthophosphate (purity 100%, analytical grade) and phosphoric acid (purity 99%, analytical grade) were obtained from Fisher, Loughborough, UK. Ammonium hydroxide (purity 100%, ACS grade) was obtained from Sigma-Aldrich, Poole, UK. Super pure water was distilled *in situ* by an Elgastat Option 4 plant.

Collection of blood and preparation of plasma

Blank whole blood was obtained by venipuncture from two healthy male volunteers who had not taken any medication during the previous 7 days. The blood was collected in tubes containing EDTA anti-coagulant and retained as whole blood (for blood cell/plasma partitioning experiments) or centrifuged to obtain plasma (for protein binding experiments).

Blank whole blood was obtained from a pool of male mice (CD-1 strain mice [code Crl: CD-1™(ICR)BR], supplied by Charles River UK Ltd) by cardiac puncture under isoflurane/oxygen anaesthesia. The blood was collected in tubes containing EDTA anticoagulant and retained as whole blood (for blood cell/plasma partitioning experiments) or centrifuged to obtain plasma (for protein binding experiments).

Mouse whole blood was pooled prior to use in blood cell/plasma partitioning experiments. Whole blood from individual human subjects was not pooled due to possible haemolysis when mixing incompatible blood groups. Whole blood was used on the day of collection.

For both species, freshly prepared separated plasma was pooled in clean glass or plastic containers and stored at about +4°C. Plasma was not used beyond a maximum storage period of 1 week at this temperature.

The pH and concentrations of total protein and albumin of each plasma pool were determined.

Human synovial fluid was obtained from patients with OA. The study protocol and related material were approved by the Local Ethics Committee of the Istituti Ortopedici Rizzoli in Bologna, Italy. The study was carried out in accordance with the current revision of the Declaration of Helsinki concerning medical research in humans, and with current Good Clinical and Laboratory Practice Guidelines (USA and EU). Synovial fluid collection was conducted by aseptic arthrocentesis from the knee joint from patients not taking any medication and stored at *ca* -20°C when not in use. The concentration of total protein in the synovial fluid sample provided was measured.

Compounds

D-[1-^{14}C]Glucosamine hydrochloride (molecular weight 215.6, specific activity 55.0 mCi/mmol [2.04 GBq/mmol], radiochemical purity 100.5%) was obtained from Hartmann Analytic and was stored at -20°C. Unlabelled crystalline glucosamine sulfate (molecular weight 573.3, purity 100.5%) was obtained from Rottapharm-Madaus and was stored at ambient temperature.

Preparation of standards and test samples

Standard solutions of D-[1-^{14}C]glucosamine hydrochloride mixed with crystalline glucosamine sulfate were freshly prepared in isotonic saline on each day required. The radiolabelled test compound (hydrochloride salt) was isotopically diluted with non-radiolabelled crystalline glucosamine sulfate in order to achieve suitable concentrations of radioactivity in the test samples. Appropriate aliquots of these solutions were spiked into whole blood and plasma of human and mouse and into human synovial fluid to give target concentrations of 400, 1000 and 4000 ng/ml, as shown in Table 1. The volume of standard solution added was the same for each sample and did not exceed 2% (v/v) of the total sample volume. Test samples were mixed gently and incubated for 10 minutes at *ca* 37°C and aliquots of each sample were taken for measurement of the radioactivity concentration.

TABLE 1

Nominal glucosamine concentrations and ratios of labelled:unlabelled glucosamine in the different experiments

Experiment	Nominal [^{14}C]glucosamine concentration (ng/ml)	Ratio of [^{14}C]glucosamine.HCl:GS	Nominal glucosamine free base concentration (ng/ml)
Protein binding	400	1 : 0	333
	1000	1 : 0	833
	4000	1 : 3	2708
Blood cell/plasma partitioning	400	1 : 50 .	252
	1000	1 : 140	626
	4000	1 : 509	2501

CGS = crystalline glucosmaine sulfate.

Radiochemical purity of D-[1-^{14}C]glucosamine hydrochloride

The radiochemical purity of D-[1-^{14}C]glucosamine hydrochloride was determined by high performance liquid chromatography (HPLC). The HLPC system consisted of an Agilent 1200 Quaternary pump with degasser G1354A and Agilent 1200 Series autosampler G1329A (Agilent Technologies UK Ltd) and a β-Ram on-line radioactivity detector (with fitted liquid flow cell) and Laura Software, Version 1.4a (LabLogic). Chromatography was performed on a Capcell Pak NH_2 80 5 μm, 250 × 4.6 mm column kept at 35°C at a flow rate of 1.5 ml/min with detection at 195 nm. The mobile phase was a 25:75 mixture of 0.02 M K_2HPO_4, adjusted to pH 7.5 with NH_4OH and H_3PO_4 and acetonitrile under isocratic conditions. Under these conditions the typical retention time for [^{14}C]glucosamine was approximately 2.6 minutes.

Procedure for plasma protein binding assessment by equilibrium dialysis

Spectra/Por® 2 dialysis membrane (Spectrum Medical Industries Inc.) of molecular weight cut-off 12,000-14,000 Daltons was prepared according to the relevant standard operating procedure and mounted onto 'Dianorm' dialysis cells. Aliquots of each test sample were loaded onto one side of the cell and aliquots of isotonic phosphate buffer, pH 7.4, were loaded onto the other side. The cells were sealed and mounted on the apparatus and dialyzed at *ca* 37°C at a constant speed between 10 and 20 rpm until equilibrium was achieved. When equilibrium had been achieved, each half of the cells was separately drained and concentrations of radioactivity were measured in the dialyzed buffer and plasma from each cell in duplicate.

Determination of non-specific binding

Using the equilibrium dialysis procedure described above, aliquots of isotonic buffer, pH 7.4, fortified with [^{14}C]glucosamine at a concentration of 400 ng/ml were loaded into each half of two dialysis cells and incubated for 6 hours at about 37°C. After incubation, the contents were drained separately from each half-cell and concentrations of radioactivity in the contents of each half of each cell were measured in duplicate.

Determination of equilibrium time and stability of [^{14}C]glucosamine under the conditions of the equilibrium dialysis procedure

Using the equilibrium dialysis procedure described above, [^{14}C]glucosamine was dialyzed in human and mouse plasma and in human synovial fluid at a concentration of 1000 ng/ml against isotonic buffer during a 16-hour period.

For each type of test sample, buffer and plasma/synovial fluid solution were drained separately from duplicate cells at 2, 6 and 16 hours and concentrations of radioactivity in the dialyzed buffer and plasma/synovial fluid solution from each cell along with the original (non-dialyzed) sample (time 0) were determined in duplicate.

Fortified (non-dialyzed) human plasma, isotonic saline and isotonic phosphate buffer, pH 7.4, incubated at 37°C for the length of time taken for equilibrium to be achieved, were deproteinised with 3 × 1 volume of ice-cold acetonitrile. The supernatants obtained by centrifugation of all three extracts were pooled, aliquots taken for measurement of radioactivity in order to calculate the extraction efficiency, and the volume of the remainder reduced under a stream of nitrogen gas at 37°C. The samples were then analyzed using the radiochromatographic method described below to assess the stability of [^{14}C]glucosamine under the conditions of the experiment.

Determination of protein binding of [^{14}C]glucosamine *in vitro*

The protein binding of [^{14}C]glucosamine *in vitro* was investigated using the equilibrium dialysis procedure described above, in human and mouse plasma and in human synovial fluid at target concentrations of 400, 1000 and 4000 ng/ml. All test samples were dialyzed in duplicate.

Procedure for blood cell/plasma partitioning assessment

Determination of equilibrium time

Duplicate aliquots of human whole blood from a single subject were fortified with [^{14}C]glucosamine at a concentration of 1000 ng/ml. The total radioactivity concentration in the fortified whole-blood sample was measured at time zero and the samples were then mixed gently and incubated at *ca* 37°C. At 5 minutes after starting the incubation, one

subsample was removed from each of the duplicate incubations and divided into two portions. Plasma was separated from one portion by centrifugation and the packed cell volume (PCV) was measured in the other portion. Concentrations of radioactivity were determined in plasma and whole blood. At 15 minutes, 30 minutes, 1 hour and 2 hours after starting the incubation, a further subsample was taken from each incubate and measurements made as detailed for the 5-minute sample (PCV, whole blood and plasma radioactivity).

Blood cell/plasma partitioning

Human (from 2 donors, separately) and pooled mouse whole-blood test samples were fortified with [^{14}C]glucosamine at each of the three target concentrations of 400, 1000 and 4000 ng/ml, mixed gently by inversion and incubated at *ca* 37°C. At the time of equilibrium (as determined above), duplicate portions of the whole blood were sampled for total radioactivity measurements, a separate sample taken for measurement of the PCV and the remainder centrifuged to obtain plasma for radioactivity assay.

Measurement of radioactivity

Aliquots of plasma, dialyzed plasma and standard solutions of [^{14}C]glucosamine were mixed with 7 ml Ultima Gold Scintillant (Perkin Elmer Life and Analytical Sciences) for measurement of radioactivity concentrations. Aliquots of each blood sample were combusted using an Automatic Sample Oxidizer (Model 307; Canberra Packard Ltd). The resultant $^{14}CO_2$ was collected by absorption in Carbosorb® E (9 ml, Perkin Elmer Life and Analytical Sciences) to which Permafluor®E$^+$ (Perkin Elmer Life and Analytical Sciences) scintillation fluid (*ca* 12 ml) was added. Combustion of standards (Spec-Chec-^{14}C; Perkin Elmer Life and Analytical Sciences) showed that recovery efficiencies were ≥97%. The results for sample combustions were corrected for efficiency accordingly.

Radioactivity was measured in all cases by liquid scintillation analysis using automatic liquid scintillation counters (Perkin Elmer Life and Analytical Sciences, formerly Wallac Oy). After selecting the optimal channel settings, quench correction curves were prepared by an external standard method using radioactive standards traceable to a National Standard. The coefficients of a quadratic quench curve

function were calculated by computer and entered into the analyzer data processors, which automatically calculated disintegration rates (dpm). The validity of the calibration curves was checked at intervals of about two weeks.

The mode of counting was pre-set at 4 minutes or until 900,000 counts had been accumulated. A solvent/scintillant 'background' disintegration rate was measured and subtracted from each sample disintegration rate.

Data processing

Concentrations of [^{14}C]glucosamine were determined from radioactivity measurements, calculated from the specific activity of D-[1-^{14}C]glucosamine hydrochloride, and the weight of crystalline glucosamine sulfate.

Protein binding

For liquid scintillation counting, the limit of quantification (gross dpm) was taken as 2 × background.

The extent (%) of binding to proteins was calculated from the relationship:

$$100(D_t - D_u)/D_t$$

where D_t is the concentration of drug (represented by total radioactivity) in the plasma after equilibrium dialysis and D_u is the unbound concentration in the dialyzate.

The mean value from the duplicate determinations was used in these calculations, providing that the values of the replicates were within 25% of the mean.

It was assumed in these analyses that the protein binding observed followed simple, reversible kinetics.

Blood cell/plasma partitioning

Concentrations of radioactivity in blood cells (C_c) were calculated from the expression:

$$[C_B - C_P(1 - PCV)]/PCV$$

where C_B and C_P represent the concentrations of radioactivity in whole blood and plasma, respectively, and PCV is the packed cell volume.

The proportion of drug in blood associated with the blood cells was calculated using the following equation:

$$\% \text{ association with blood cells} = 100[1 - R(1 - PCV)]$$

where R is the plasma:whole blood concentration ratio for total radioactivity.

RESULTS

Radiochemical purity

The radiochemical purity of D-[1-^{14}C]glucosamine hydrochloride was determined both prior to and at the end the experimental phase, and was 100.5% and 94.0%, respectively (data not shown).

Protein binding

Determination of non-specific binding

There was no evidence of non-specific binding of [^{14}C]glucosamine (at a nominal concentration of 400 ng/ml) to the equilibrium dialysis apparatus; a value of 1.2% was obtained.

Stability

The stability of [^{14}C]glucosamine was determined in isotonic saline, isotonic phosphate buffer, pH 7.4, and in human plasma. It was found that [^{14}C]glucosamine was stable for up to 16 hours in all of the above matrices. For the matrices analyzed, the purity was found to be 100.4%, 100.2% and 95.9%, respectively.

Determination of protein binding

Concentrations of [^{14}C]glucosamine in plasma and the extent of binding to human and mouse plasma proteins in addition to the proteins of human synovial fluid at test material concentrations of 400, 1000 and 4000 ng/ml are presented in Tables 2 and 3, respectively. It was found that there was effectively no protein binding in either

TABLE 2

Mean binding of [^{14}C]glucosamine to the proteins in plasma pooled from two male human subjects and from male mice *in vitro*, incubated for 2 hours

Species	[^{14}C]Glucosamine nominal concentration (ng/ml)	Total [^{14}C]glucosamine concentration in human plasma after dialysis (ng/ml)	Unbound [^{14}C]glucosamine concentration in buffer after dialysis (ng/ml)	Protein binding (%)
Human	400	207	214	-3.3
		199	209	-5.0
	1000	502	548	-9.1
		489	514	-4.9
	4000	2027	2099	-3.6
		2056	2095	-1.9
Mouse	400	210	215	-2.4
		209	216	-3.2
	1000	474	482	-1.8
		485	502	-3.6
	4000	2015	2063	-2.4
		2056	2140	-4.1

TABLE 3

Binding of [^{14}C]glucosamine to proteins in human synovial fluid *in vitro*, incubated for 2 hours

[^{14}C]Glucosamine nominal concentration (ng/ml)	**Total [^{14}C]glucosamine concentration in human synovial fluid after dialysis** (ng/ml)	**Unbound [^{14}C]glucosamine concentration in buffer after dialysis** (ng/ml)	**Protein binding** (%)
400	216	210	3.0
	201	203	-1.0
1000	507	512	-1.0
	518	521	-0.6
4000	2173	2182	-0.4
	1420	1455	-2.5

human or mouse plasma or human synovial fluid. There were essentially no concentration-dependent differences in binding in either species for plasma or for human synovial fluid.

Blood cell/plasma partitioning

A 30-minute equilibration time for [^{14}C]glucosamine incubated with human whole blood was selected as optimal, based on the results of preliminary experiments (data not shown). The blood cell/plasma partitioning of [^{14}C]glucosamine was determined using whole blood from two male human subjects and pooled whole blood from mice. Parameters determined included concentrations of test material in plasma and blood cells, in addition to their associated blood:plasma concentration radioactivity ratio (with its reciprocal) and the calculated association of radioactivity with blood cells. Mean values are presented in Table 4. In the male human samples, it was found that the amount of [^{14}C]glucosamine that bound to blood cells was in the range 43-47%. For mouse whole blood, it was determined that the range for binding was 27-29%. For both human and mouse blood cell/plasma partitioning there was no consistent concentration-dependent effect displayed for the concentration range investigated.

DISCUSSION AND CONCLUSIONS

The results obtained in the present study indicate that [^{14}C]glucosamine does not bind to human plasma or synovial fluid proteins or to mouse plasma proteins. These results are clinically relevant as they were obtained using glucosamine concentrations ranging from 333 to 2501 ng/ml, i.e. in the same range as those determined at steady state in the plasma of healthy volunteers and in plasma and synovial fluid of patients with OA receiving repeated therapeutic oral doses of CGS of 1500 mg once a day /13,14/. Therefore, in humans after administration of crystalline glucosamine sulfate, the unbound (pharmacologically active) fraction of glucosamine at the site of action (the joint) is the same as that in plasma and direct comparison of the two concentrations is possible. In addition, plasma protein binding displacement drug-drug interactions are unlikely during the clinical use of crystalline glucosamine sulfate when this drug is co-administered with other drugs even if these are highly

TABLE 4

Mean parameters associated with the determination of concentrations of radioactivity in plasma, blood cells and blood, blood:plasma radioactivity concentration ratios and calculated percentage of radioactivity associated with blood cells following incubation of [^{14}C]glucosamine with blood from two male human volunteers separately and from pooled male mice, for 30 minutes

Species	Nominal concentration (ng/ml)	PCV	Concentration in plasma (ng/ml)	Concentration in blood cells (ng/ml)	Blood:plasma radioactivity concentration ratio	Plasma:blood radioactivity concentration ratio	Association of radioactivity with blood cells (%)
Human	400	0.463	504.7	503.0	0.998	1.002	47
	1000	0.457	1034.0	921.1	0.965	1.058	43
	4000	0.481	4295.3	3510.9	0.907	1.109	43
Mouse	400	0.429	544.1	309.7	0.788	1.271	28
	1000	0.429	1528.2	768.0	0.787	1.277	27
	4000	0.427	5806.1	3535.4	0.804	1.244	29

Experiments were conducted in duplicate.
PCV = packed cell volume

bound to plasma proteins. Finally, no corrections for the unbound drug fraction are needed when comparing human and mouse pharmacokinetic or distribution data.

Blood cell/plasma partitioning experiments showed that partitioning of radioactivity in human and mouse plasma and blood was similar to the measured PCV, indicating similar distribution of glucosamine in plasma and blood cells. Results were similar across the 10-fold concentration range used in the present study. Therefore, no preferential accumulation of glucosamine occurs in human and mouse blood cells and, therefore, no correction for the blood-to-plasma concentration ratio is needed to assess glucosamine total blood clearance from plasma data in either species.

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