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High-performance liquid chromatographic–mass spectrometric analysis of oligosaccharides from enzymatic digestion of glycosaminoglycans

Application to human samples☆

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ABSTRACT

Glycosaminoglycan contents were evaluated in plasma and urine samples from volunteers treated intravenously with a mixture of dermatan sulphate and heparin, combining a novel liquid chromatographic–mass spectrometric technique for the determination of oligosaccharides from glycosaminoglycans with a classical technique for the extraction of glycosaminoglycans from biological samples (precipitation with cetylpyridinium chloride). In plasma samples dermatan sulphate and heparin can be measured for 2 h after treatment; urine excretion was detectable for 24 h. These results suggest that this novel approach is promising for future studies on the pharmacokinetics of glycosaminoglycans, although some technical aspects need further improvement, mainly regarding the procedures for sample clean-up; cetylpiridinium precipitation is a complex procedure and the recovery is limited.

INTRODUCTION

Sulphated glycosaminoglycans (GAGs) are a large family of heterogeneous polysaccharides playing important roles in all living organisms and sometimes having notable pharmacological activities [1]. Pharmacokinetic studies of these products have generally been carried out by evaluating biological effects (*e.g.*, coagulation) [2] or by means of radiolabelled derivatives [3]. Both approaches present problems. GAG derivatives sometimes present biological effects, such as antithrombotic activity,

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that cannot be evaluated easily *in vitro* [4]. The use of radiolabelled molecules involves ethical problems that reduce the applicability of this method. Few pharmacokinetic studies have been performed based on chemical analysis to detect GAGs in biological fluids (generally electrophoresis was used) [5,6].

Recently we developed a new technique for the determination of GAGs using high-performance liquid chromatography–mass spectrometry (HPLC–MS) [7], with a sensitivity and specificity suitable for its application to biological samples. This method is based on the degradation of GAGs present in real samples with specific lyases followed by determination of the disaccharides, typical of different GAGs, by HPLC–MS. An ionspray interface was adopted for effective ionization of such polar molecules. In this paper we describe the results obtained with this technique on blood and urine samples from healthy volunteers treated intravenously (i.v.) with a mixture of heparin (Hep) and dermatan sulphate (DeS). GAGs were precipitated from biological samples with cetylpyridinium chloride (CPC) as reported elsewhere [6,8,9].

EXPERIMENTAL

Chemicals

Hep, DeS, heparinase (Hep.ase), chondroitinase ABC (Ch.ase ABC) and pronase were purchased from Sigma. DeS of different average molecular weight (3300, 5000, 7100, 12 000 and 20 000 dalton) for gel permeation chromatographic (GPC) calibration were kindly provided by Ricerchimica (Milan, Italy). Acetonitrile (HPLC-grade) and all other chemicals, of the purest grade available, were obtained from Fluka. Ultrapure water was obtained with a Milli-Q system (Millipore). The internal standard, deuterium-labelled DeS, was prepared as described previously [7]. Briefly, an aliquot of DeS was treated at 105°C for 5 h with hydrazine to achieve partial (>50%) deacetylation then, after purification by dialysis and lyophilization, the sample was reacetylated with an excess of perdeuterated acetic anhydride. The content of deuterium in the internal standard, determined by HPLC–MS on enzymatic digestion samples, was 40%. The GAG mixture, for the treatment of volunteers, was obtained as vials for clinical use (Ateroid; Crinos, Villa Guardia–Como, Italy).

Standard solutions

Stock solutions of 1 mg/ml Hep and DeS were prepared in water and stored at −20°C; these solutions were prepared freshly each week. Solutions of deuterated DeS were also prepared with the same technique for use as an internal standard.

Extraction from plasma

A 2-ml volume of each plasma sample were treated for 12 h at 45°C with 6.0 mg of pronase to degrade the protein matrix, after addition of 0.4 ml of 50 m*M* Tris–acetate buffer (pH 8.0) containing the internal standard (10 μg of deuterium-labelled DeS). Then trichloroacetic acid (50%, w/w, in water) was added up to a final concentration of 5% and the samples were left for 1 h at 4°C. After centrifugation at 2000 *g* (Hermle BHG Z320K) for 10 min, the supernatants were collected and 4.0 *M* NaOH was added to obtain a final concentration of 0.5 *M*. After 24 h at 4°C, the samples were neutralized with 10.0 *M* HCl and dialysed for 48 h against distilled water with continuous recycling at room temperature using dialysis membranes with a molecular weight cut-off of 3500 (Spectra/Por molecular porous membrane tubing; Spectrum) prewashed in distilled water. After lyophilization (Univapo 15A H; Stepbio), the samples were dissolved in 0.2 ml of 20 m*M* NaCl and injected into the HPLC system to separate high-molecular-weight GAGs from low-molecular-weight degradation products by means of GPC.

Separations were carried out with the technique described below and for each sample the fraction eluting between 9.5 and 16.5 min was collected. GAGs were precipitated from the mobile phase by addition of CPC to a final concentration of 0.015% and overnight incubation at 4°C. Then the samples, after thawing at 20°C, were centrifuged for 20 min at 3000 *g* at room temperature. The precipitates obtained, after carefully removal of supernatants, were washed three times with 80% ethanol saturated with NaCl, three times with 80% ethanol in water, once with absolute ethanol and once with diethyl ether. Finally, the dried pellets obtained from precipitation were digested for 48 h at 37°C with 1.5 mU of Hep.ase and 80 mU of Ch.ase ABC dissolved in 0.5 ml of 50 m*M* ammonium acetate buffer (pH 7.0) containing 1 m*M* $CaCl_2$ as activator of the enzymatic reaction. Then enzymes were precipitated by

addition of three volumes of cold methanol, centrifugation (10 min at 5000 *g*) and the supernatants were lyophilized. Before HPLC–MS analysis the samples were dissolved in 50 μl of distilled water.

Sample volumes of 25 μl were injected into the RP-HPLC system connected to a mass spectrometer (PE-Sciex API III biomolecular mass analyser) equipped with an ionspray interface without splitting, and the traces corresponding to the molecular weights of the disaccharides from enzymatic digestion of Hep and chondroitin sulphate (ChS) are analysed.

Extraction from urine

Aliquots of urine samples (10.0 ml) spiked with 500 μg of deuterated internal standard, after centrifugation (15 min at 5000 *g*) to remove any particulates, were treated with CPC, at a final concentration of 1%, to precipitate GAGs. Then samples were treated as described for plasma.

Chromatographic separations

All the HPLC separations were performed using a Gilson HPLC system (Model 302 pump + Model 305 pump) equipped with a microbore dynamic mixer and a Rheodyne Model 7125 injection valve with a loop of 500 μl for GPC separations or a loop of 20 μl for HPLC–MS analysis with a microbore column. When needed, UV absorbance was monitored using a Pharmacia Model 2141 variable-wavelength UV–VIS detector and data were treated with a Varian 4270 computing integrator.

GPC separations were performed with a Pharmacia TSK G2000 SW column (300 mm × 7.5 mm I.D.) connected with a Supelco Progel TSK G3000 PWXL column (300 mm × 7.8 mm I.D.) under isocratic conditions with 0.1 *M* NaCl as mobile phase at a constant flow-rate of 1.0 ml/min; the signal was monitored at 210 nm.

Gradient RP-HPLC separations were obtained with a hexyl reversed-phase column (Phase Separations C_6, 5 μm, 150 mm × 1 mm I.D.). The mobile phase was composed of 3.3 m*M* tetrapropylammonium hydroxide (TPA) in water adjusted to pH 4 with formic acid (buffer A) and 3.3 m*M* TPA in water–acetonitrile (10:90) at the same pH (buffer B). The gradient elution conditions adopted consisted of 3 min isocratic at 100% buffer A, then a linear gradient to 40% buffer B in 17 min and a final isocratic step of 10 min at this composition. The flow-rate was 0.05 ml/min.

Mass spectrometry

The effluent from the HPLC microbore column, flow-rate 0.05 ml/min, was connected to a PE-Sciex API III triple quadrupole mass spectrometer (Sciex, Thornhill, Canada) equipped with an atmospheric pressure articulated ionspray source. Analyses were performed by selective ion monitoring for negative ions, with a dwell time of 150 ms, at *m/z* 458, 461 and 946. These values, according to our previous results, correspond respectively to the molecular ions of the disaccharide produced by the enzymatic digestion of DeS, the molecular ions of the disaccharide characteristic of the enzymatic digest of deuterated DeS (internal standard) and the ions formed by the adducts of trisulphated disaccharide, typical of Hep enzymatic digestion, with the ion-pair reagent (TPA).

Calibration and quantification

Blank plasma samples (2.0 ml) from a pool of plasma and blank urine samples (20.0 ml) were spiked with both Hep and DeS, using stock solutions, at concentrations of 1, 3, 30 and 100 μg/ml. Appropriate volumes of water were also added to ensure an equivalent total volume (*e.g.*, 0.2 ml for plasma and 2.0 ml for urine) in each instance. For quantification these samples were treated as unknown samples. Separate for calibration graphs for Hep and DeS were constructed of GAG concentrations *versus* the ratio of the peak areas of Hep or DeS to that of the Internal standard. Peak areas for DeS, Hep and the internal standard were evaluated for the peaks eluting at 13.8 min with *m/z* 458, 17.8 min with *m/z* 946 and 13.8 min with *m/z* 461, respectively. Calibration graphs for a new set of spiked samples were prepared after every 30 unknown samples to avoid quantification bias due to the complex extraction and degradation procedure.

Recovery and reproducibility

The percentage recovery of each GAG was determined by comparing the extraction from plasma or urine spiked with GAGs (1, 10 and 100 μg/ml of each GAG) with the equivalent concentration of standard solution dissolved in water, lyophilized and degraded with enzymes as real samples. In both

instances internal standard was added. The reproducibility of the method was determined from samples prepared as before and analysed in triplicate within the same series of analyses or in different set of samples.

Application to plasma and urine samples after i.v. treatment with a mixture of GAGs

Two normal female volunteers were treated intravenously with a single dose of a GAG mixture (DeS 30 mg + Hep 70 mg). Venous blood samples (10.0 ml) were taken before and then 10, 20, 30, 60, 240 and 360 min after the treatment. The blood samples were collected in tubes containing sodium citrate as anticoagulant and immediately centrifuged at 2000 *g* for 10 min at 4°C; 4 ml of each sample were stored at −80°C until analysed.

Urine samples were collected 24 h before and after drug treatment, divided into fractions of 6 h (0–6, 6–12, 12–18 and 18–24 h). After measuring the volume of the diuresis, 50 ml of each fraction were stored at −80°C until analysed.

RESULTS

Chromatographic separations

Using the GPC conditions described previously, standards of DeS, Hep and deuterated DeS eluted together with a retention time of 11 min; the peaks were large, from 9.5 to 17.5 min, owing to the heterogeneity of these molecules. The standard of low-molecular-weight DeS (3300 dalton) had a retention time of 16.7 min and the total elution time of the peak was between 14 and 18 min. Based on these results, a collection time between 9 and 16.5 min was chosen in prepartive GPC to isolate polymeric GAGs (moleculr weight > 3000 dalton) from oligosaccharides. Fig. 1 shows a chromatogram obtained under reversed-phase separation conditions with mass spectrometric detection for an enzymatic digestion of standard GAGs. As reported previously [7], the disaccharide characteristic of DeS degradation, at *m/z* 458, elutes between 13 and 14 min (13.31 min); the same oligosaccharide from the internal standard has the same retention time, as shown by the trace at *m/z* 461. The trisulphated disaccharide characteristic of Hep is more retained and is eluted between 17 and 18 min (17.41 min).

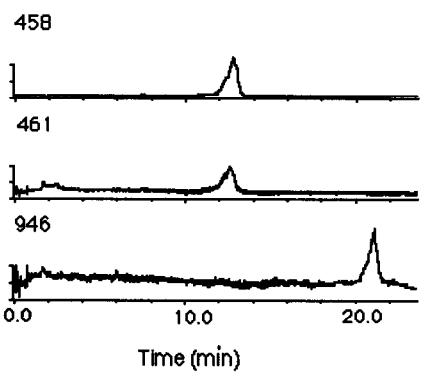

Fig. 1. Chromatographic traces of oligosaccharides from an enzymatic digestion of a standard containing DeS (*m/z* 458), deuterated DeS (*m/z* 461) and Hep (*m/z* 946). The amount injected corresponds to 1 μg of each substance.

Linearity

Good linear correlations were observed between concentrations and peak areas of standard DeS or Hep in water. Straight lines were also obtained by plotting peak-area ratios of DeS or Hep to the internal standard *versus* concentrations in plasma or urine (Fig. 2). For plasma and urine, the relationships were linear ($r > 0.995$) over the range of concentrations tested (1–100 μg/ml) for both DeS and Hep. For Hep the regression line intercept was not significantly different from zero whereas for DeS, especially in urine, the intercept was higher than zero, probably owing to the endogenous ChS and DeS [6,10].

Precision and accuracy

Standards in water of Hep and DeS at 1–10 and 100 μg/ml were analysed in triplicate, after enzyme digestion, to evaluate the variability of the methods without the extraction problems. The results were similar at different concentrations with both substances and the relative standard deviations were between 2.8 and 3.3%. Table I show the concentrations and standard deviations determined for spiked plasma and urine samples, at the same concentrations, extracted and analysed in triplicate within the same set of samples, using the same calibration graphs. Similar relative standard deviations, ranging from 3.3 to 4.5%, were observed with Hep and DeS at different concentrations in both plasma and urine. Table I also gives the results obtained with the same analytical technique and identical

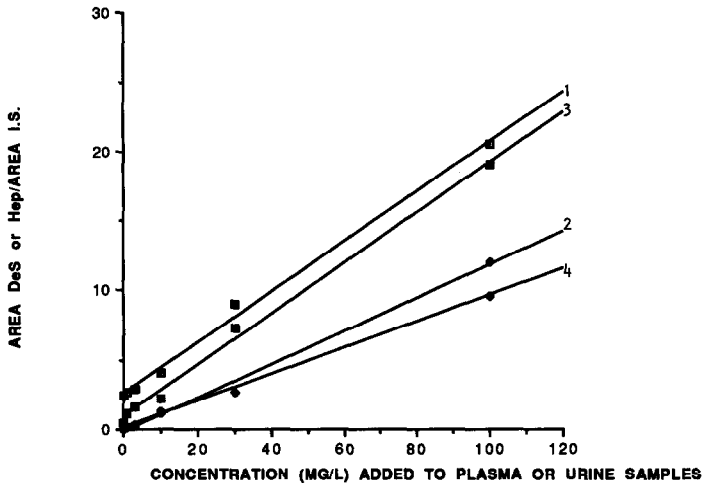

Fig. 2. Linear regressions between concentration of Hep or DeS added to samples of plasma or urine and ratio of peak area of Hep or DeS to that of deuterated DeS added as internal standard (I.S.). Lines: 1 (□) = urine + DeS, R^2 = 0.995; 2 (◆) = urine + Hep, R^2 = 0.995; 3 (■) = plasma + DeS, R^2 = 0.995; 4 (◇) = plasma + Hep, R^2 = 0.997.

range of concentrations from samples analysed in triplicate on different days then using different calibration graphs. The relative standard deviations are higher, between 4.3 and 5.6%, but still reasonable.

Extraction recoveries

Table II gives the results for experiments on the recovery of GAGs and internal standard in plasma and urine. The recoveries of DeS and Hep added to plasma were similar for both molecules, 27.7 ± 2.3

TABLE I

EVALUATION OF PRECISION AND ACCURACY FOR REPLICATE (n = 3) SPIKED SAMPLES OF PLASMA OR URINE ANALYSED ON THE SAME DAY OR ON DIFFERENT DAYS

Concentration added to sample (mg/l)	Concentration determined (mg/l)		Relative standard deviation (%)	
	Hep	DeS	Hep	DeS
Plasma samples analysed on the same day				
100	98.6	101.3	3.9	3.3
10	10.7	9.6	4.1	3.6
1	0.9	1.1	4.2	4.3
Plasma samples analysed on different days				
100	97.4	100.8	4.4	4.3
10	9.1	9.8	5.2	4.8
1	1.1	1.1	5.3	5.1
Urine samples analysed on the same day				
100	103.4	96.2	3.6	4.1
10	10.5	11.1	3.9	4.3
1	0.9	1	4.5	4.5
Urine samples analysed on different days				
100	96.8	102.1	4.3	3.9
10	9.7	10.6	4.5	4.6
1	0.9	1.1	5.6	5.1

TABLE II

EXTRACTION RECOVERIES AND RELATIVE STANDARD DEVIATIONS FOR REPLICATE STANDARDS (n = 3) EXTRACTED FROM SPIKED HUMAN PLASMA OR URINE

Samples	Concentration mg/l	Recovery (%)			Relative standard deviation (%)		
		DeS	Hep	Deuterated DeS	DeS	Hep	Deuterated DeS
Plasma	100	30.2	32.1	31.6	3.4	3.8	3.1
	10	27.3	28.9	30.5	4.4	3.6	3.9
	1	25.6	27.2	27.8	3.8	4.5	4.3
Urine	100	19.2	18.9	19.8	2.8	2.6	2.4
	10	17.3	18.3	3.1	3.5	2.8	
	1	17.1	16.8	17.8	3.1	3.9	3.3

and 29.4 ± 2.5%, respectively (mean ± standard devition at different concentrations tested), with small differences depending on the concentration tested. In urine samples a lower recovery was observed with both GAGs, 17.9 ± 1.2 and 17.8 ± 1.1%, respectively, at all concentrations. The recovery of the internal standard was of the same order as those of Hep and DeS.

Selectivity

No endogenous interfering peaks, except the possible physiological presence of the same substances, were visible in blank plasma or urine samples at the retention times and m/z values characteristic of disaccharides from enzymatic digestion of DeS and Hep.

Sensitivity

The detection limits in plasma samples, corresponding to a signal-to-noise ratio of 2, were 0.5 μg/ml for DeS and 1 μg/ml and Hep. In urine samples, under the same experimental conditions, the detection limits were 0.1 μg/ml for DeS and 0.2 μg/ml for Hep.

Pharmacokinetic study

Fig. 3 shows the chromatograms at m/z 458–946 of plasma samples withdrawn from a volunteer before and 10–120 min after treatment with the mixture of GAGs. The peak corresponding to the internal standard, m/z 461, was well characterized in all samples (chromatograms not reported). In the chromatogram at m/z 458 a peak with the character-

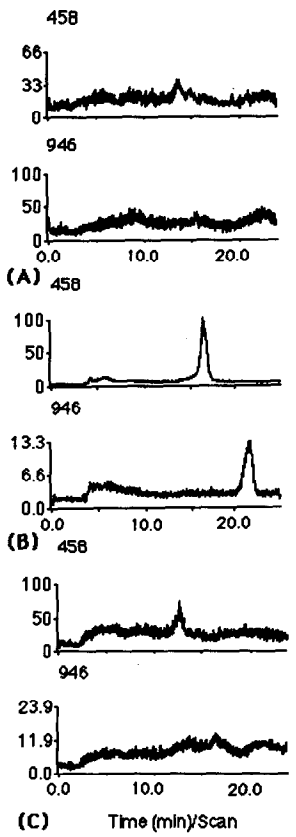

Fig. 3. Chromatographic profiles corresponding to oligosaccharides from DeS (m/z 458) or Hep (m/z 946) obtained from plasma samples of a volunteer (A) before and (B) 10 min and (C) 120 min after i.v. treatment with a mixture of GAGs.

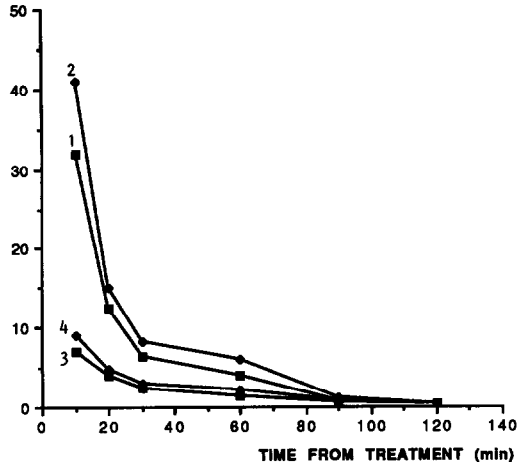

Fig. 4. Plot of DeS and Hep concentrations measured in plasma samples withdrawn from two volunteers at different times after i.v. treatment with a mixture of GAGs. Lines: 1 (□) = patient A, Hep; 2 (◆) = patient B, Hep; 3 (■) = patient A, DeS; 4 (◇) = patient B, DeS.

istic retention time of the digestion product of DeS or ChS is present in the sample before the treatment due to the endogenous content of these GAGs. As expected, its area increased in the samples after treatment. In contrast the peak corresponding to the digestion product of Hep (*m/z* 946, retention time 17.4 min) can be observed only after treatment; in fact a previous study did not show detectable levels of endogenous Hep in blood [6].

Fig. 4 shows graphically the plasma levels of DeS and Hep evaluated in volunteers after the treatment with GAGs. The concentrations of DeS reported were subtracted from the basal levels of DeS of 5.4 and 4.8 μg/ml in patients A and B, respectively. Detectable levels of both GAGs were measured for 2 h after the treatment, the plasma half-life being short, about 10 min.

Fig. 5 shows the chromatograms at *m/z* 458, 461 and 946 of urine samples collected before and 0–6 or 18–24 h after the treatment. DeS is present at high concentration also in the blank urine, but after intravenous treatment the concentrations are higher. Hep could not be measured before treatment but considerable amounts were present after drug administration.

Results for urine excretion are summarized in Table III (results for DeS were subtracted from the basal values). Considerable amounts of DeS and

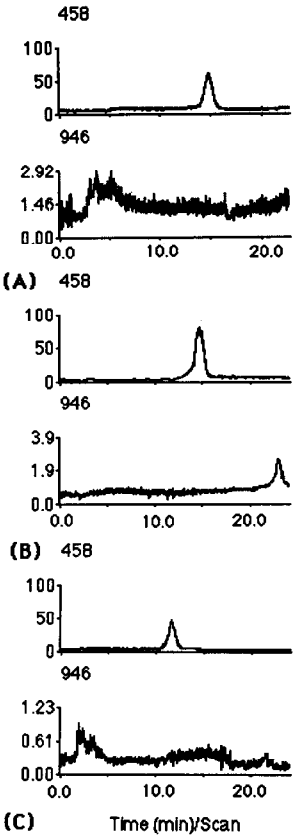

Fig. 5. Chromatographic profiles corresponding to oligosaccharides from DeS (*m/z* 458) or Hep (*m/z* 946) obtained for urine samples from a volunteer collected (A) before and (B) 0–6 h and (C) 18–24 h after i.v. treatment with a mixture of GAGs.

TABLE III

AMOUNTS OF DeS AND Hep EXCRETED IN URINE OF TWO VOLUNTEERS COLLECTED AT DIFFERENT TIMES AFTER I.V. TREATMENT WITH A MIXTURE OF GAGs

Treatment	Time after treatment (h)	Amount determined (mg excreted)	
		Patient A	Patient B
Hep	0–6	9.3	8.0
6–12	4.1	4.5	
	12–18	0.9	0.8
	18–24	0.0	0.0
DeS	0–6	4.4	3.9
	6–12	3.6	4.6
	12–18	1.2	1.5
	18–24	0.6	1.2

Hep are excreted in the 24 h after the treatment, about 35–40% of the injected dose. In the last fractions Hep is not present, suggesting a faster metabolism of this substance than DeS.

DISCUSSION

An effective technique has been developed for studying GAGs in biological samples with adequate sensitivity for pharmacokinetic studies. The technique is specific (no interference was observed in blank samples), is reproducible (reasonable inter- and intrassay variability) and quantification is linear over a wide range of concentrations. The results of recovery studies were not very good, although they were reproducible, and were far from the values reported for some studies using similar techniques [6,9]; moreover this technique is fairly complex. We consider that in future studies, other extraction techniques, *e.g.*, on ECTEOLA-cellulose [5] or Polybrene-Sepharose [3], should be tested in order to improve the recovery and the sensitivity of the technique. The use of degration of GAGs by specific lyases gave interesting results and is highly specific. The lack of activity, at least for Hep.ase [11], on desulphated products may be a limitation for future studies aimed also at the study of possible metabolites that seem to be desulphated [12]. The introduction of other degradation techniques such as with nitrous acid may be appropriate.

The present HPLC–MS approach is really promising in attempts to study GAG metabolites, mainly for the structural information obtainable. Moreover, the results obtained with deuterium-labelled GAGs as internal standard suggest the use of similar derivatives in healthy volunteers to study the pharmacokinetics without interferences from endogenous GAGs. With the improvements suggested above we consider that this technique should be effective also for more demanding pharmacokinetic studies on GAGs such as those after oral treatment or concerning drug metabolism.

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