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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR METHYLGLYOXAL BIS(GUANYLHYDRAZONE) (METHYL GAG) IN PLASMA AND URINE

KENNAN C. MARSH, A. J. REPTA and LARRY A. STERNSON*

Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kan. 66045 (U.S.A.)

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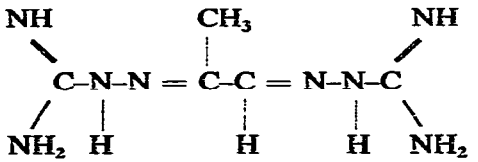
SUMMARY

A clinically useful analytical method is described for monitoring both plasma and urine levels of methylglyoxal bis(guanylhydrazone) administered in the clinical management of certain neoplasms. The drug is initially separated from the biological matrix by retention on a small (2 cm) column packed with weak cation-exchange resin. The analyte is subsequently eluted quantitatively from the column with hydrochloric acid, and then separated and quantitated by paired ion high-performance liquid chromatography on an RP-18 column. The drug is detected to levels of 50 ng/ml of biological fluid by monitoring the column effluent spectrophotometrically at 280 nm. Absorbance was linearly related to drug concentration over the range 50 ng–50 μg/ml of plasma or urine, and measurements could be made with a precision of ± 4% over this range.

INTRODUCTION

Shortly after Freelander and French[1] described the growth inhibitory effect of methylglyoxal bis(guanylhydrazone)** (methyl GAG; NSC-32946) (I), it was found to have significant activity against acute myelocytic leukemia. On a daily dosage schedule the toxic effects were often dose limiting and life threatening[2–4]. The mechanism of antitumor action of methyl GAG has not been elucidated, but evidence indicates that the physiological effects may be primarily related to the inhibition of the enzyme S-adenosyl methionine decarboxylase which catalyzes the synthesis of spermidine[5].



(I)

* To whom correspondence should be addressed.

** IUPAC name is 1,1′-[(methylethanediylidene)dinitrilo]diguanidine dihydrochloride monohydrate.

More recent investigations with methyl GAG have shown favorable responses in several types of solid tumors and lymphomas, with acceptable toxicity, when patients are dosed at lower levels on a weekly schedule[6,7]. Since the drug is now undergoing extensive clinical testing, a method was sought to monitor methyl GAG levels in plasma and urine. In this report we describe such a clinically useful method based on initial separation of the drug from the biological matrix by cation-exchange chromatography and subsequent high-performance liquid chromatography (HPLC) analysis of the drug.

EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A pump, Model U6K injector, and Model 440 absorbance detector operated at 280 nm. Peak areas were measured with a Varian Assoc. (Palo Alto, Calif., U.S.A.) Model 111C chromatography data system interfaced with the absorbance output of the detector. Optimization of the mobile phase composition was accomplished with the addition of a second pump and a Waters 660 solvent programmer. The column used was a Waters μBondapak C_{18} (30 cm $\times$ 3.9 mm I.D.; 10 μm particle size).

UV spectra were recorded in 1-cm quartz cells using Cary (Palo Alto, Calif., U.S.A.) Model 118 and 219 spectrophotometers.

Materials

Methyl GAG was used as obtained from the National Cancer Institute (Bethesda, Md., U.S.A.). The sodium salts of pentane, hexane, heptane and octane sulfonic acids were purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.). Sodium dodecanesulfonate was purchased from Aldrich (Milwaukee, Wisc., U.S.A.). All of the sodium alkyl sulfonates were used as received. 5-Hydroxytryptamine (Serotonin) was obtained from Sigma (St. Louis, Mo., U.S.A.) as the anhydrous oxalate salt. Methanol was Fisher HPLC grade. All other chemicals were reagent grade. Cation-exchange resin, Bio-Rex 70 (200–400 mesh) was purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.) in the sodium form. All water was distilled in glass following mixed-bed deionization. Glass disposable transfer pipets (9 in. long) were purchased from Curtin Matheson Scientific (Houston, Texas, U.S.A.).

Plasma was purchased (Community Blood Center, Kansas City, Mo., U.S.A.) as "recovered human plasma" containing citrate–phosphate–dextrose anticoagulant and was stored at 5°. Fresh urine was obtained from healthy human volunteers and was stored overnight in ice.

Methods

pK_a Determinations. The apparent pK_a values of methyl GAG dihydrochloride were determined by titrating an aqueous sample of methyl GAG dihydrochloride (0.01 *M*) under nitrogen with 0.5-ml portions of 0.1 *M* aqueous potassium hydroxide at ambient ($\approx$ 20°) temperature according to the method of Albert and Sergeant[8].

Separation of methyl GAG from plasma and urine. Disposable columns were

prepared by slurry packing *ca.* 0.2 g of dry Bio-Rex 70 cation-exchange resin (suspended in water) into 9-in. glass transfer pipets to give 22 × 5 mm wet packed columns. Freshly prepared columns were washed sequentially with water (5 ml), 0.5 *M* sodium acetate solution (adjusted to pH 4 with concentrated hydrochloric acid) (5 ml), 0.5 *M* hydrochloric acid solution (5 ml), and pH 6 hydrochloric acid solution (2–4 ml).

Plasma samples were adjusted to pH 6 with 25 μl of 1 *M* hydrochloric acid per ml of plasma. A 1-ml volume of pH-adjusted plasma (or pH 6 hydrochloric acid solution) containing methyl GAG was applied to the ion-exchange column, followed by washings with 5 ml of water and 5 ml of 0.5 *M* sodium acetate solution (pH 4). The methyl GAG was then eluted from the column with 1.9 ml of 0.5 *M* hydrochloric acid. The eluent from this final wash was collected in 2 ml volumetric flasks containing 40 μl of a solution of the internal standard (5-hydroxytryptamine, 0.571 mg/ml in pH 4 hydrochloric acid) and was then adjusted to volume with 0.5 *M* hydrochloric acid. Sodium hydroxide (80 μl of 12 *M* solution) was added to each sample in preparation for chromatographic analysis.

Urine samples (1 ml) were applied directly (without pH adjustment) to the ion-exchange columns, then washed with water (6 ml) and subsequently carried through the same steps described for processing plasma samples.

Chromatography

The acidic ion-exchange column eluent containing methyl GAG was subsequently chromatographed as an ion pair on an octadecylsilane bonded phase column using C_5–C_8 and C_{12} sodium alkyl sulfonates as hetaerons with UV absorbance detection of the effluent at 280 nm. In each case the sodium alkyl sulfonate concentration was 2.5 m*M* and the concentration of acetic acid was 0.5%. The methanol–water concentrations were varied using a solvent programmer.

The mobile phase used for final analysis consisted of 2.5 m*M* sodium pentane sulfonate and 0.5% of glacial acetic acid in methanol–water (17.5:82.0). The flow-rate was maintained at 2.0 ml/min. Injection volumes of 20 μl were used for all HPLC analyses.

Quantitative analysis

Methyl GAG was quantitated by determining the analyte: internal standard (5-hydroxytryptamine) peak area ratio on a Varian CDS 111C computing integrator and comparing this value with a standard curve constructed after analysis of plasma or urine samples containing a known amount of the drug. A standard curve for plasma was generated by analyzing three samples of plasma at each of 12 different concentrations of methyl GAG ranging from 0.141–50.2 μg/ml. Samples for this curve were prepared by the addition of 5–89 μl of aqueous acidic solutions (pH 4) of methyl GAG [2.82 mg/ml (or 0.28 mg/ml)] to plasma, to give a final volume of 5 ml [10 ml for the 140 and 282 ng/ml samples]. Standard curves in urine were prepared by analysis of three samples at each of six different concentrations of methyl GAG ranging from 0.28–5.6 μg/ml of urine. To permit evaluation of recovery, a third standard curve was constructed by spiking mobile phase [methanol–water (17.5:82.0) containing 0.5% glacial acetic acid and 2.5 m*M* sodium pentane sulfonate] with methyl GAG and internal standard at the same concentrations used in the generation

of the plasma and urine curves and then immediately subjecting these solutions to direct HPLC analysis.

Clinical sample processing. Patients judged suitable for this clinical protocol were administered methyl GAG (700 mg/m²) intravenously as a 25-min infusion. Blood samples were drawn in polypropylene syringes at time intervals immediately before and 17, 40, 75, 180 min, 5 h, 8 h, 24 h, and 48 h after initiation of infusion. Plasma was produced by centrifugation of these samples in polystyrene tubes at 1300 *g* for 4 min. Recovered plasma was stored in polystyrene tubes in ice until processing ($\leqslant$72 h).

RESULTS AND DISCUSSION

Separation of methyl GAG from biological fluid matrices

Direct extraction of plasma or buffer (pH 7.4) with water immiscible solvents of varying properties (*i.e.* polarity, proton-donating and proton-accepting abilities, etc.) resulted in no measurable partitioning of methyl GAG into the organic layer. The apparent pK_a values of methyl GAG dihydrochloride were determined titrimetrically to be $pK_{a_1} = 7.63 \pm 0.04$ and $pK_{a_2} = 9.05 \pm 0.01$. Adjustment of solutions containing methyl GAG to pH 11 (where the drug should exist in non-ionic form), prior to extraction, did not improve extraction efficiency above 10%, apparently due to the pronounced hydrophilicity of the analyte, even in its uncharged form. Extraction of methyl GAG from potassium carbonate-saturated solutions with low-molecular-weight alcohols improved extractability to *ca.* 50%, but the precision of the procedure was poor. This was presumably due to increased degradation of the analyte at the high pH created by carbonate saturation. Furthermore, extraction with low-molecular-weight alcohols was non-specific resulting in co-extraction of many substances from plasma and urine causing subsequent difficulties in HPLC analysis (resolution) of the drug.

An ion-exchange method for the isolation of radioactive methyl GAG described by Oliverio *et al.*[9] was adapted for clinical use with small volume biological samples.

The applicability of cation-exchange resins (Bio-Rex 70) as the support media to separate (and retain) the drug from biological matrices was investigated. Due to the high viscosity of biological fluids, resins of small mesh (25–50 and 100–200) were initially examined, but were found to be inefficient in retaining 1 ml samples containing methyl GAG (5 μg/ml) even where $>$ 1 g of dry resin was used. However when 0.2 g (dry weight) of this resin in the smallest mesh size commercially available (200–400), was used, quantatitive retention of methyl GAG was realized in all biological samples tested over the concentration range 0.14–60 μg/ml. This amount of dry resin (0.2 g) offers a 20,000 fold excess capacity over that theoretically necessary for retention of the largest plasma level (50 μg) of methyl GAG/ml anticipated[9] after i.v. infusion of the drug at doses of $\leqslant$ 1 g/m². The resin bed was pre-washed (see Experimental) to facilitate settling, remove salts and convert the resin from the sodium to proton form.

Methyl GAG could be removed from the ion-exchange column with 1.9 ml of 0.5 *M* hydrochloric acid. Larger volumes of eluent were required when less concentrated acids were used or with acids more concentrated than 2 *M*. The elution

behavior of 0.5 and 1 *M* hydrochloric acid solutions was similar. The 0.5 *M* acid was chosen to minimize the equivalents of base needed to raise the pH of the samples to pH $\approx$ 3 prior to HPLC analysis. This partial neutralization —carried out with sodium hydroxide solution (12 *M*) to minimize the volume change when added to the 2-ml samples— is required to prevent the deterioration of the silica-based columns used in the final analysis.

Plasma samples were adjusted to pH 6 with 1 *M* hydrochloric acid to insure that methyl GAG completely protonated prior to ion-exchange processing. Plasma samples not adjusted to pH 6 gave wide variability in duplicate analysis. After application of the sample, washing of the resin with 5 ml of water in the case of plasma and 6 ml in the case of urine followed by 5 ml of aqueous sodium acetate solution (0.5 *M*,

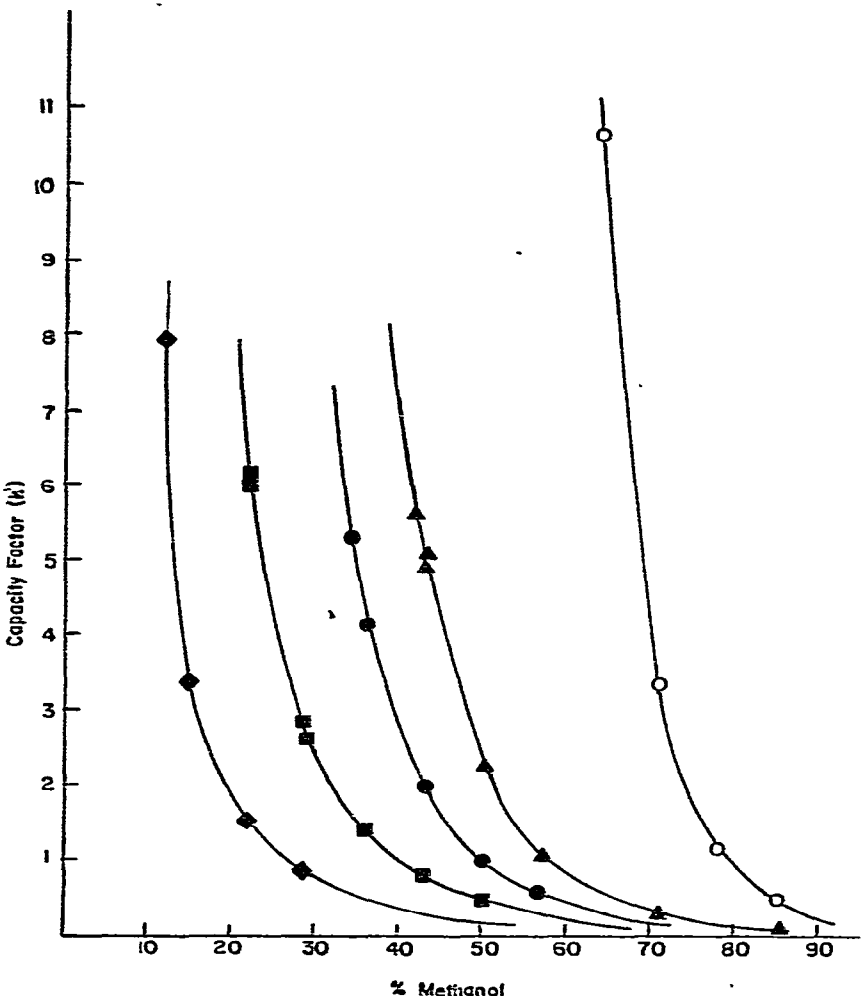


Fig. 1. Capacity factor (k') for methyl GAG *vs.* methanol concentration in mobile phase containing 0.5% acetic acid and 2.5 m*M* sodium alkyl sulfonate. Sodium pentane (◆); hexane (■); heptane (●); octane (▲); and dodecane (○) sulfonate.

pH 4) was found to be adequate to remove the extraneous substances from the ion-exchange column. HPLC monitoring of the wash solutions obtained from an aqueous sample of methyl GAG applied to the column showed no drug in the effluent.

Chromatography

Due to its high molar absorptivity ($\varepsilon = 40{,}000$) at 283 nm, spectrophotometric monitoring at 280 nm after separation by HPLC was considered a favorable approach to quantitation of methyl GAG. Initial attempts using both normal- and reversed-phase chromatography were unsuccessful until an ion-pairing agent was added to the mobile phase. All of the alkyl sulfonates studied (C_5–C_8) were found to be effective hetaerons for the chromatographic separation of methyl GAG when the methanol–water ratio was appropriately adjusted and acetic acid concentration was maintained at 0.5%. Fig. 1 shows the effect of the addition of methanol to the mobile phase on retention of methyl GAG. From the shape of these curves, regions can be selected where small incremental changes in methanol concentration have large effects on the capacity factor, k', and therefore chromatographic separations. Table I shows the system efficiency achieved with each of the alkyl sulfonic acids. Maximum efficiency is achieved with sodium pentane sulfonate which was then chosen as the counter ion. In a mobile phase methanol–water (17.5:82.0) which was 2.5 mM in pentane sulfonate and 0.5% in acetic acid, methyl GAG was rapidly eluted from the column (retention volume 12 ml) and was well resolved (resolution > 1.5)[10] from the internal standard (retention volume 8 ml) peak and any plasma or urine constituents co-eluted from the ion exchanger as seen in Fig. 2.

TABLE I

NUMBER OF THEORETICAL PLATES (N) FOR INDIVIDUAL CHROMATOGRAPHIC SYSTEMS AT $k' = 2.85$ WITH $CH_3(CH_2)_nSO_3Na$ HETAERONS

$N = 16[t_r/t_w]^2$, where t_r = retention time, t_w = peak width at baseline. $k' = (t_r - t_u)/t_u$, where t_u = time of elution of unretained species. 20 μl of 10^{-5} M methyl GAG injected onto C_{18} column with mobile phase 2.5 mM alkyl sulfonate, 0.5% acetic acid in methanol–water of composition to yield k' (methyl GAG) = 2.85.

n	N
4	1500
5	1440
6	1380
7	1180

Standard curves and sensitivity

In construction of standard curves in plasma, the ratio of the areas of the methyl GAG/internal standard peaks was related linearly to the ratio of the concentration of methyl GAG (μg/ml)/concentration of internal standard (μg/ml) over the concentration range 0.141–50.20 μg methyl GAG/ml of plasma. Linearity of responses was determined by least squares analysis of data points and gave the line $y = 0.66x - 0.97$ with a zero order correlation coefficient of 0.995. Detection limits were approximately 50 ng/ml of plasma as determined by direct analysis of drug supplemented plasma samples with a standard deviation of less than $\pm$ 5% (as determined for triplicate samples). Total recovery of methyl GAG from plasma and urine samples was

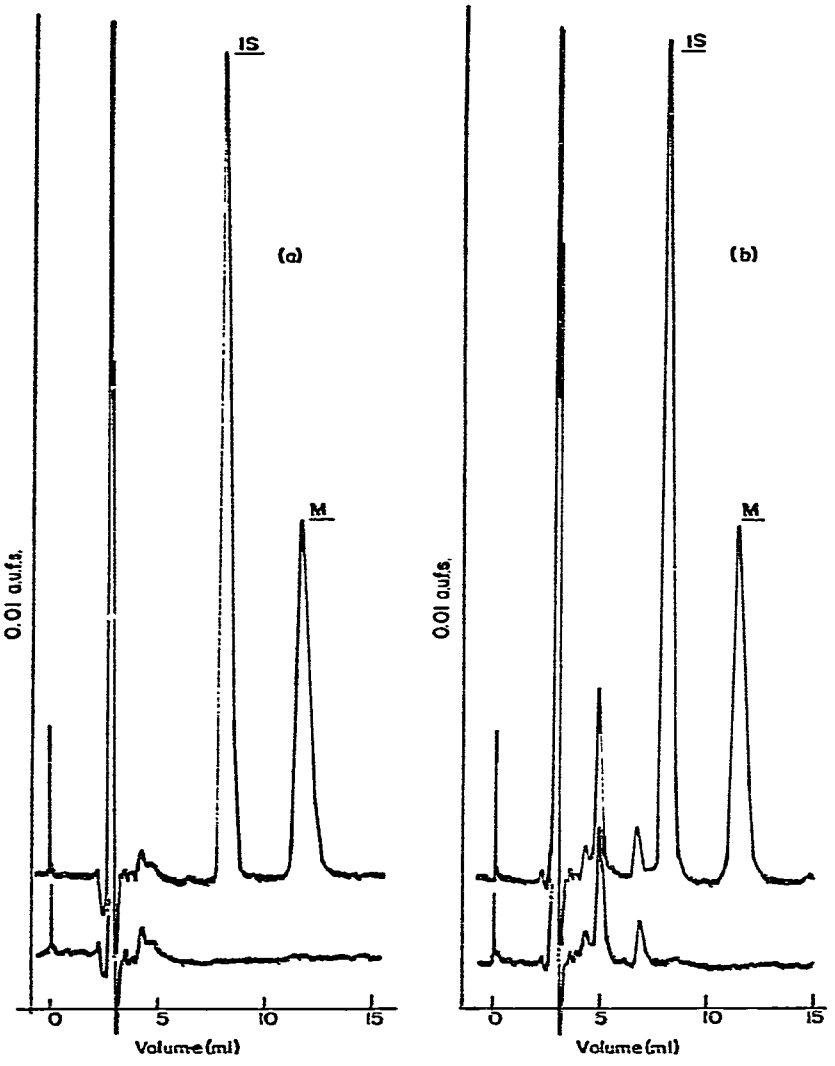


Fig. 2. Chromatogram of methyl GAG from (a) plasma and (b) urine. Methyl GAG (M) retention volume: 12 ml; 5-Hydroxytryptamine (IS) retention volume: 8 ml. Separation carried out on octadecylsilane bonded phase column with 0.5% acetic acid and 2.5 m*M* sodium pentane sulfonate in methanol–water (17.5:82.0) as the mobile phase. Flow-rate, 2 ml/min. In each case, the lower tracing represents a blank, *i.e.* biological fluid not containing drugs, but carried through the analysis sequence.

evaluated by constructing a standard curve by spiking mobile phase with methyl GAG at the same 12 concentrations used in the preparation of the plasma-based standard curve and directly subjecting these samples to HPLC analysis. The resulting line is represented by the equation $y = 0.67x + 0.35$ (with a correlation coefficient of 0.999). Comparison of this curve with that generated for plasma samples indicated that recovery varies from 91–104% over the concentration range of 0.05–50 μg/ml.

The standard curve from urine samples was found to be linear from 0.28–5.6

μg methyl GAG/ml of urine. Using the same ratios as described for the plasma standard curve, regression analysis for these points generates the line $y = 0.62x + 0.60$, with a correlation coefficient of 0.997. Detection limits were the same as in plasma samples with a standard deviation of ± 4%. Recovery monitored in urine ranged from 91–97%, with a coefficient of variance never exceeding ±5%.

Clinical monitoring of methyl GAG. Stability studies carried out directly in plasma and urine indicated that samples were stable for more than 3 months when stored at 0°. Some degradation was noted at elevated pH (pH ⩾ 10). Erratic adsorption of methyl GAG to glass container surfaces was observed, and therefore clinical samples were stored in polystyrene vials.

The clinical utility of this analytical method is shown in Fig. 3, which shows plasma levels of methyl GAG determined after the drug was infused (700 mg/m²)

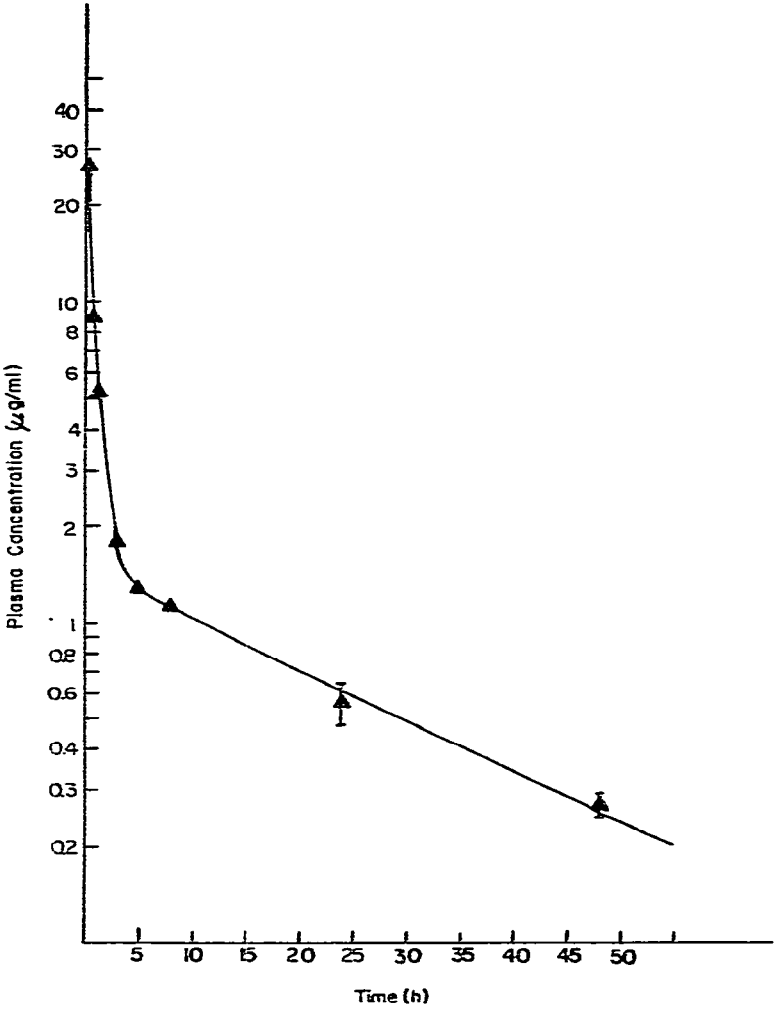


Fig. 3. Plasma levels of methyl GAG present in a female patient administered the drug intravenously (700 mg/m²) over a 25-min period in the treatment of renal cell carcinoma.

intravenously over a 25-min period to a female patient suffering from renal cell carcinoma. Plasma clearance half-life was calculated to be 16 h with a volume of the central compartment of 60 l. Cumulative urinary excretion of methyl GAG is shown in Fig. 4.

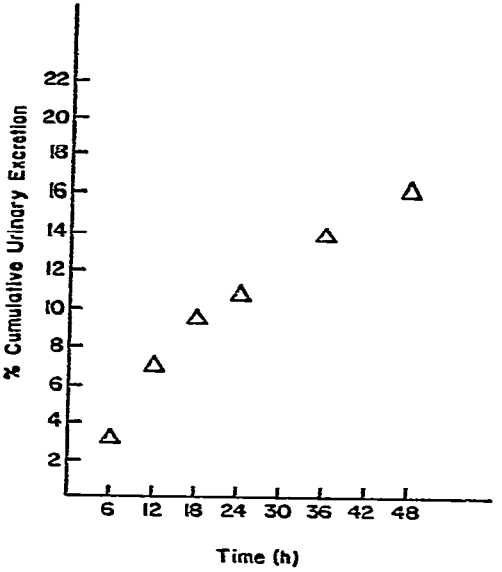


Fig. 4. Cumulative urinary excretion profile for the patient described in Fig. 3.

We have presented a rapid, sensitive method for the analysis of methyl GAG in both plasma and urine samples. Its clinical applicability and potential utility have been demonstrated. This method is currently being utilized in pharmacokinetic studies.

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