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Note

Determination of the carcinogen methylazoxymethyl-β-D-glucosiduronic acid in rat bile and urine

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Methylazoxymethanol (MAM), the aglycone of cycasin (methylazoxymethyl-β-D-glucopyranoside), administered by any route is carcinogenic to rodents. Carcinomas of the colon are induced in rats upon multiple injections of MAM[1–3]. Injected MAM may be conjugated with glucuronic acid in the liver to form MAM–β-D-glucosiduronic acid (MAM–GlcUA) and excreted with the bile. The MAM–GlcUA moves to the lower intestinal tract and is hydrolyzed by bacterial β-glucuronidase to give the free carcinogen MAM[4].

A sensitive analytical method for the determination of MAM–GlcUA in the bile and urine could be used to establish whether or not MAM injected into an animal is conjugated with glucuronic acid. This paper describes a gas chromatographic (GC) procedure for the quantitative analysis of MAM–GlcUA in bile and urine.

MATERIALS AND METHODS

Chemicals

Methylazoxymethyl-β-D-glucosiduronic acid was prepared by catalytic oxidation of cycasin[5]. MAM was prepared by the method of Kobayashi and Matsumoto[2].

Amberlite XAD-2 resin (non-ionic, polymeric adsorbent; Mallinckrodt, St. Louis, MO, U.S.A.) was purified by extraction with acetone, followed by thorough rinsing with water. Darco G-60 activated charcoal (Matheson, Coleman & Bell, Norwood, OH, U.S.A.) and Celite analytical filter-aid (Johns-Manville, Denver, CO, U.S.A.) were prewashed with several volumes of 95% ethanol and water before use[6]. Tril-Sil concentrate (Pierce, Rockford, IL, U.S.A.) was stored in a desiccator under refrigeration until used. Reagent grade pyridine was distilled over KOH. The silylation reagent was prepared by introducing one volume of Tri-Sil concentrate and four volumes of dry pyridine into a 30-ml serum bottle capped with a PTFE-lined septum[7].

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Gas chromatography

The XAD-2 column was prepared by pouring 5–7 cm of prewashed resin into a glass chromatography tube (15 cm × 15 mm I.D.). The charcoal column was prepared by pouring a water slurry of 7 g of prewashed Darco G-60–Celite (1:1, w/w; thoroughly premixed) into a chromatographic column (25 cm × 17 mm). After rinsing each column with four volumes of water, the XAD-2 column was placed atop the charcoal column. The effluent end of the charcoal column was inserted through a rubber stopper which was placed in a filter-flask. The flask was connected to a vacuum source and a slight vacuum was applied to the eluate receiver during a chromatographic run.

Gas chromatographic analysis was carried out on a Bendix 2500 equipped with a flame ionization detector. Peak areas were determined by an on-line Autolab Vidar 6300 digital integrator. The column was a U-tube (190 cm × 2 mm I.D.) packed with 1.5% OV-17 on Gas-Chrom Q (60–80 mesh). Operating conditions were: column temperature, 230°C; detector temperature, 240°C; injector temperature 240°C; nitrogen flow-rate, 25 ml/min.

A 2-ml volume of silylation reagent was transferred with a syringe into a glass vial, which contained the dried MAM–GlcUA, and capped with a PTFE-lined screw cap. The mixture was stirred with a vortex mixer for 30 sec and allowed to remain at room temperature for at least 1 h prior to GC analysis. Samples of 0.5–2.0 μl were directly injected into the gas chromatograph[8].

Urine and bile collection

Male Wistar rats (250 ± 10 g) were injected intraperitoneally with measured amounts of MAM–GlcUA and placed individually in metabolism cages. Urine was collected in a test-tube which was immersed in ice contained in a small Dewar flask. Control urine was collected from non-injected rats. The common bile duct of male rats was cannulated, the animals were placed in restrainers and bile was collected in small vials.

RESULTS

Gas chromatography of MAM–GlcUA standard

Gas chromatographic analysis of the pertrimethylsilylated derivative of MAM–GlcUA produced a chromatogram with a symmetrical peak against a clean background (Fig. 1). Excellent linearity was obtained for 1-μl injections of standards with concentrations in the 0.09–4.7 mg/ml range. The reproducibility of the first several determinations was excellent, but the sensitivity changed with successive analyses. Thus, it was necessary to construct a standard curve each day and check it at intervals throughout the day. Another problem, that of great fluctuation in detector sensitivity, was alleviated by repacking the inlet end of the column every day. A dark brown deposit derived from the urine and bile samples formed at the inlet after a number of MAM–GlcUA determinations evidently interfered with the analysis. The detector also had to be cleaned about every 2 weeks to remove the build-up of silicon from the silylation reagent and of carbon from pyridine.

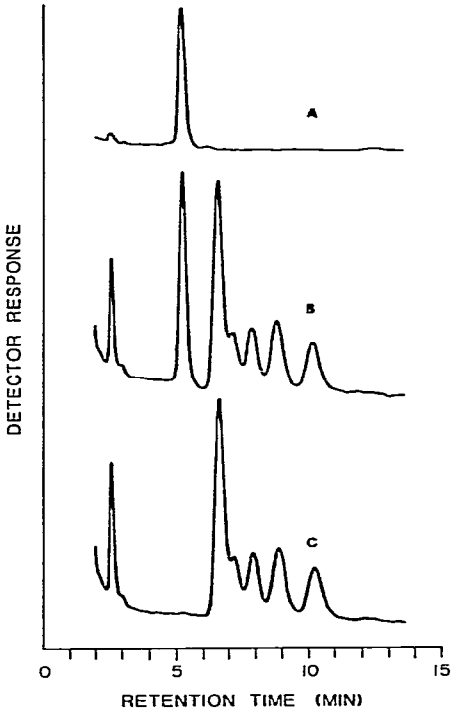

Fig. 1. Gas chromatograms of pertrimethylsilylated MAM–GlcUA (A), MAM–GlcUA added to rat urine (B) and rat urine (C). Operating conditions: column temperature, 230°C; detector temperature, 240°C; inlet temperature, 240°C; nitrogen flow-rate, 25 ml/min.

Gas chromatography of MAM–GlcUA added to urine

Direct determination of MAM–GlcUA in urine after lyophilization of the sample was found to be unsatisfactory, because of the large amount of salt residue which prevented the use of small volumes of silylation reagent. Therefore, a clean-up procedure was devised to remove the salts and lipophilic contaminants in the urine (see Materials and methods). The urine sample was poured through the XAD-2 column and the percolate was allowed to drip directly onto the top of the Darco–Celite bed. The XAD-2 bed was washed with 100 ml water and removed. The Darco–Celite column, through which the 100 ml water had passed, was then eluted with 100 ml of 75% aqueous ethanol.

The alcoholic eluate, which contained the MAM–GlcUA, was evaporated to dryness under vacuum in a Buchi rotary evaporator at 60°C. The solid residue was triturated with 7 ml methanol in three portions and each was filtered through a 5 mm pore size, Mitex membrane filter to remove particulate impurities. The filtrate, collected in a glass vial, was evaporated to dryness under a stream of nitrogen on a warm heating plate. The residue was exhaustively dried in a vacuum desiccator and then silylated.

The chromatogram of control urine subjected to the purification procedure displayed several large peaks (Fig. 1C), but was clear at the retention position of MAM–GlcUA. Urine samples to which MAM–GlcUA was added yielded a chromatogram with a cleanly resolved MAM–GlcUA peak (Fig. 1B).

TABLE I

RECOVERY OF MAM–GLUCOSIDURONIC ACID ADDED TO RAT URINE

The indicated amount of MAM–GlcUA was added to rat urine and the compound was separated by carbon column chromatography and eluted with 75% aqueous ethanol. The dried compound was derivatized with Tri-Sil reagent and the quantity determined by GC.

MAM–GlcUA added (mg)	*Recovery (%) ±S.D. (n = 10)*
	99.0 ± 1.1
2.0	99.7 ± 1.1
5.0	100.5 ± 1.2
10.0	83.3 ± 2.7

Four different quantities, 1, 2, 5 and 10 mg, of MAM–GlcUA were added to 1 ml of control rat urine and the samples were analyzed. The recoveries were good for the three smaller quantities, but recovery dropped for the 10-mg level (Table I). This was probably due to overloading of the column, and thus for experimental work when the quantity was greater than 5 mg a smaller aliquot of the collected urine was analyzed.

Gas chromatography of MAM–GlcUA in bile

Direct pertrimethylsilylation and GC analysis of MAM–GlcUA in bile residue was satisfactory. MAM–GlcUA was added to bile and the mixture was transferred to a small vial and lyophilized. A 2-ml volume of silylation reagent was added to the bile residue in the vial, thoroughly mixed and allowed to remain at room temperature for at least 1 h. A 1-μl volume of the silylated mixture was injected into the gas chromatograph. There were no interfering peaks in the vicinity of the MAM–GlcUA peak.

Four different quantities of MAM–GlcUA were added to 1-ml samples of control bile. Each quantity was determined in triplicate. The recoveries were uniform and averaged at least 93% (Table II).

TABLE II

RECOVERY OF MAM–GLUCOSIDURONIC ACID ADDED TO RAT BILE

The indicated amount of MAM–GlcUA was added to bile and the mixture was lyophilized. The compound was pertrimethylsilylated in the bile residue and the quantity of the derivatized compound determined by GC.

MAM–GlcUA added (mg)	*Recovery (%) (n = 4)*
0.075	95.5*
0.15	93.3
0.50	93.8
1.00	96.3

* Mean from four determinations.

Determination of MAM–GlcUA in bile and urine of rats injected with the compound and MAM

Bile and urine from male Wistar rats injected with MAM–GlcUA were analyzed to determine the detectable amounts. Four dosage levels of MAM–GlcUA were intraperitoneally injected into rats with and without a bile duct cannula and analyzed for quantities of the compound excreted in the bile and urine. Six animals per dosage level were used for urine collection over 24 h, and two animals per dosage level for bile collection over 12 h. In addition, four male rats with bile duct cannula were injected with 20 mg free MAM per kg bodyweight and the bile was collected. The urine was made up to 25 ml and suitable aliquots were analyzed for MAM–GlcUA. A 1-ml volume of the collected bile was used per determination. No MAM–GlcUA was found in the bile. The recoveries of injected MAM–GlcUA in the urine ranged from 95 to 103%. The injected MAM–GlcUA was essentially quantitatively excreted in the urine (Table III).

TABLE III

RECOVERY OF MAM–GLUCOSIDURONIC ACID FROM URINE AND BILE OF RATS INJECTED WITH MAM OR MAM–GlcUA

Compound injected	*Amount (mg per kg bodyweight)*	*Recovery (%)*	
		*Urine**	*Bile***
MAM–GlcUA	40	100.3 (95–102)	0.0
MAM–GlcUA	80	102.9 (97–104)	0.0
MAM–GlcUA	160	95.3 (95– 96)	0.0
MAM–GlcUA	320	98.5 (98– 99)	0.0
MAM	20		0.0***

* Mean from six urine samples. Figures in parentheses indicate range of values.
** Two bile samples for each dosage level of MAM–GlcUA, except where stated otherwise.
*** Four bile samples.

DISCUSSION

Trace quantities of glucuronides, which have small aglycones and are hence highly water-soluble, are difficult to quantitate in biological fluids. Extraction of glucuronides with water-immiscible solvents would be highly desirable since they would remove the bulk of materials which interfere with the quantitation procedure[8]. Steroid glucuronides can be extracted with solvents of high polarity, but very polar steroid glucuronides are not completely extracted[9]. Amberlite XAD-2, a synthetic polystyrene polymer, Sephadex LH-20 and anion-exchange resins have been used for the isolation of high-molecular-weight glucuronides from biological fluids[10]. MAM–GlcUA in urine was not adsorbed by Amberlite XAD-2. In addition, Sephadex LH-20, Sephadex G-10, anion-exchange resin and reversed-phase high-performance liquid chromatography failed, for different reasons, to separate satisfactorily MAM–GlcUA from interfering substances.

The method developed for the determination of MAM–GlcUA offers the advantage of combining high accuracy and sensitivity with brevity of analysis time.

MAM–GlcUA is eluted from a carbon–Celite column with water–ethanol after the bulk of the impurities have been removed with water. The XAD-2 column, while it does not adsorb MAM–GlcUA, removes other substances in the urine which interfere with the GC analysis. The determination of MAM–GlcUA in urine is accurate when the amount of the compound passed through the Darco–Celite column is less than 5 mg, but the recovery drops to 80% when 10 mg of MAM–GlcUA are present. The quantitation of MAM–GlcUA at any concentration can accurately be carried out by utilizing a suitable aliquot of the urine collected. The determination of MAM–GlcUA after direct pertrimethylsilylation of lyophilized bile is sensitive and as low as 0.075 mg/ml of MAM–GlcUA can be determined.

The quantitative recovery of MAM–GlcUA in the urine of rats injected with various amounts of the compound demonstrated that the method is accurate. The slightly less than 100% recovery in some of the urine samples may have been due to incomplete excretion of the compound by rats in 24 h. The observation that no MAM–GlcUA was detected in the bile of animals injected with MAM or MAM–GlcUA is indicative that MAM is not conjugated with glucuronic acid. It is unlikely that minute quantities of MAM–GlcUA excreted with the bile had gone undetected. If a 350-g rat were injected with 160 mg MAM–GlcUA per kg bodyweight and if only 0.5% of the injected compound were excreted in as much as 3.5 ml bile, the compound could be detected with greater than 90% accuracy. A greater percentage of injected compound would be expected to be present if excretion of MAM–GlcUA with the bile was a major route for the elimination of the compound.

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