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DETERMINATION OF MELENGESTROL ACETATE IN BOVINE TISSUES BY AUTOMATED COUPLED-COLUMN NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method has been developed for the determination of melengestrol acetate in bovine tissues at lower levels than previously reported. Liquid–liquid extraction of tissue homogenates provided crude clean-up while final isolation, screening, and quantification was done on-line with an automated, normal-phase, coupled-column high-performance liquid chromatographic system. The chromatographic system included phenyl and silica analytical columns for the purposes of isolation and final separation, respectively These columns provided a large difference in selectivity when operated under normal-phase conditions which allowed for the efficient isolation of melengestrol acetate from the complex tissue extracts. Mobile phases werre composed of hexane and dichloromethane modified with methanol and water. Transfer and enrichment of the analyte from the primary phenyl column to the silica column was via a short (12 mm × 4 mm I.D.) silica column. Regeneration and equilibration of the phenyl column was performed after the injection of each tissue extract and was accomplished simultaneously while analytical separation occurred on the final silica column. Routing of the mobile phases and regeneration solvent was performed with automated switching valves The total time required for each analysis was 12 min. Quantification is demonstrated using external standards with UV detection at 287 nm. The overall recovery of the method was 86% with a coefficient of

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variation of 9.84% at the 10 ppb [the American billion (10^9) is used in this article] level in bovine liver extracts.

INTRODUCTION

Melengestrol acetate (17α-hydroxy-6-methyl-16-methylene pregna-4,6-diene-3,20-dione acetate, MGA; Fig. 1) is a synthetic progestational anabolic steroid which is administered as a feed additive to feedlot heifers for growth promotion and to suppress estrus [1]. The use of MGA has been approved by the Food and Drug Administration since 1968 [2]. Regulatory efforts have focussed on monitoring the residue levels of MGA that can accumulate in the edible tissues of food-producing animals. This requires analytical methodology capable of detecting and confirming trace levels (less than 5 ppb) of these residues in very complex biological matrices such as liver tissues. The official method for monitoring MGA has a detection limit of 25 ppb with final detection by electron-capture gas chromatography (GC) [3]. Because there is a zero tolerance level for MGA in uncooked edible bovine tissues [4] the minimum requirement of a new method for the determination of MGA included a limit of detection (LOD) of 5 ppb in liver with mass spectral (MS) confirmation.

The chromatographic capability of a single high-performance liquid chromatographic (HPLC) or GC separation procedure is often inadequate for the analysis of complex environmental or biological samples such as bovine liver. Sample pre-treatment or clean-up steps are usually required to remove the majority of endogenous components and provide concentration of the analyte prior to analysis. The method described herein utilizes two HPLC columns coupled together as a two-dimensional chromatographic approach to the analysis of complex samples that allowed sample clean-up to be performed on-line with analytical separation and detection.

The successful application of column switching in HPLC was facilitated by the implementation of switching valves which have a low hold-up volume and are capable of operating under high pressure. This was first reported by Huber et al. [5]. Lankelma and Poppe [6] have described the chromatographic parameters which determine the concentration (i.e. enrichment) factor of a coupled-column HPLC system which ultimately defines the efficiency of the system. Numerous reports have described the concept and potential of coupled-column HPLC including those of Majors [7], Freeman [8], Roth et al. [9], Ogan and Katz [10],

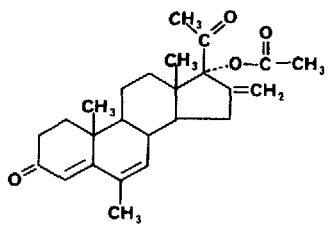


Fig. 1. Structure of melengestrol acetate (MGA).

Little et al. [11], Ramsteiner and Bohm [12], Lecaillon et al. [13], and Raglione et al. [14]. Giddings [15] has described the capabilities and limitations of two-dimensional separation systems, in general, including coupled-column HPLC.

MGA is a neutral, lipophilic steroid which is difficult to isolate by manual sample clean-up techniques such as solid-phase extraction (SPE) [16–18]. The selectivity and resolution afforded by SPE was not sufficient to isolate this analyte from complex bovine tissue extracts. By utilizing a conventional LC column with 3-μm particles for the primary clean-up step instead of SPE the isolation of MGA from the tissue matrix was achieved. Coupling a second analytical column to this primary 'clean-up' column allowed on-line screening, quantification with external standards, and sample purification suitable for subsequent GC–MS analysis.

EXPERIMENTAL

Materials

Analytical standards of MGA were provided by the United States Department of Agriculture–Food Safety Inspection Service. Benzene was purchased from Burdick & Jackson (Muskegon, MI, U.S.A.). All other solvents were HPLC grade from Fisher Scientific (Rochester, NY, U.S.A.). Heptafluorobutyric acid anhydride (HFAA) was purchased from Aldrich (Milwaukee, WI, U.S.A.).

Administration

Incurred tissues were provided from an administration of MGA to three heifers each weighing between 230 and 455 kg. The doses were 0.5, 1.5, and 3.0 mg per head per day by capsule for seven days with a 24-h withdrawal time before slaughter. Incurred muscle, liver, and kidney tissues were collected, homogenized, and stored at $-20°C$ until the time of analysis.

Tissue homogenization and extraction

Control tissues were purchased locally and homogenized in 500-g batches with a blender (Waring, VWR Scientific, Rochester, NY, U.S.A.). Aliquots of 5 g of bovine liver, muscle, or kidney homogenates were then vortex-mixed with 10 ml of water after which 20 ml of acetonitrile were added followed by mixing on a vortex mixer for 30 s. The homogenates were then mixed by rotation (30 rotations/min) for 30 min, then centrifuged (250 000 *g*, 20 min). The supernatant (30 ml) was then transferred to a clean 150 mm$\times$25 mm I.D. test-tube. Hexane (8 ml) and dichloromethane (2 ml) were added and mixed by rotation (30 rotations/min) for 2 min. Samples were then centrifuged (45 000 *g*, 2 min). A three-phase liquid system was obtained and MGA partitioned into the middle layer which consisted primarily of acetonitrile. The middle layer (16 ml) was transferred to a 20-ml scintillation vial and evaporated under nitrogen at 60°C which produced ca. 90 mg of an oily residue.

The liver and kidney extracts were each dissolved in 500 μl of cyclohexane and the muscle extracts in 200 μl of cyclohexane. The dissolved extracts were filtered through 0.45-μm nylon microfilter-fuge tubes (Rainin, Woburn, MA, U.S.A.). The filtrates (50 μl) were then subjected to LC analysis.

Chromatographic system

Dissolved tissue extracts were injected using a Model ISS-100 autosampler with a 50-μl sample loop (Perkin-Elmer, Norwalk, CT, U.S.A.). Sample clean-up and regeneration of the primary 'clean-up' column and final analytical separation were performed on three columns coupled together by two Waters air-actuated automatic switching valves (Waters Assoc., Milford, MA, U.S.A.) as shown in Fig. 2. The mobile phases and regeneration solvent were delivered by three Waters HPLC pumps (Model 510 and 6000A). A fixed-wavelength (254 nm) UV detector (Waters, Model 440) was used for monitoring the retention time of MGA on the primary phenyl column. The last column was connected to a UV detector operated at 287 nm (Model 783, Kratos Analytical, Ramsey, NJ, U.S.A.). A temperature control module (Waters) maintained columns 1 and 3 at 30°C. The pumps and actuation of the switching valves and integrator (Model 3390A, Hewlett-Packard, Avondale, PA, U.S.A.) were controlled by a Waters automated gradient controller (Model 680).

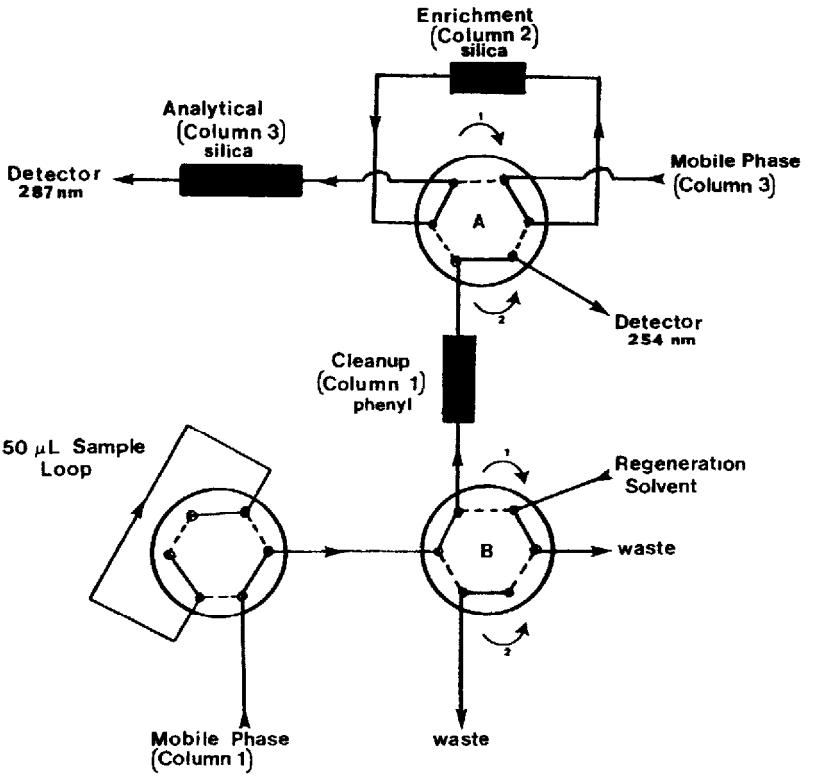


Fig. 2. Schematic of coupled-column HPLC system for isolating MGA. In position 1 switching valve A re-routed the effluent from the phenyl 'clean-up' column (column 1) to the silica enrichment column (column 2) to achieve the collection of MGA. Switching valve A was programmed to return to position 2 when collection was complete Eluent for the silica analytical column (column 3) then eluted MGA from the enrichment column onto column 3 for final separation. The regeneration of column 1 began with switching valve B in position 1 and continued for 4 min. In position 2 switching valve B re-routed mobile phase back to column 1 for equilibration which took at least 4 min.

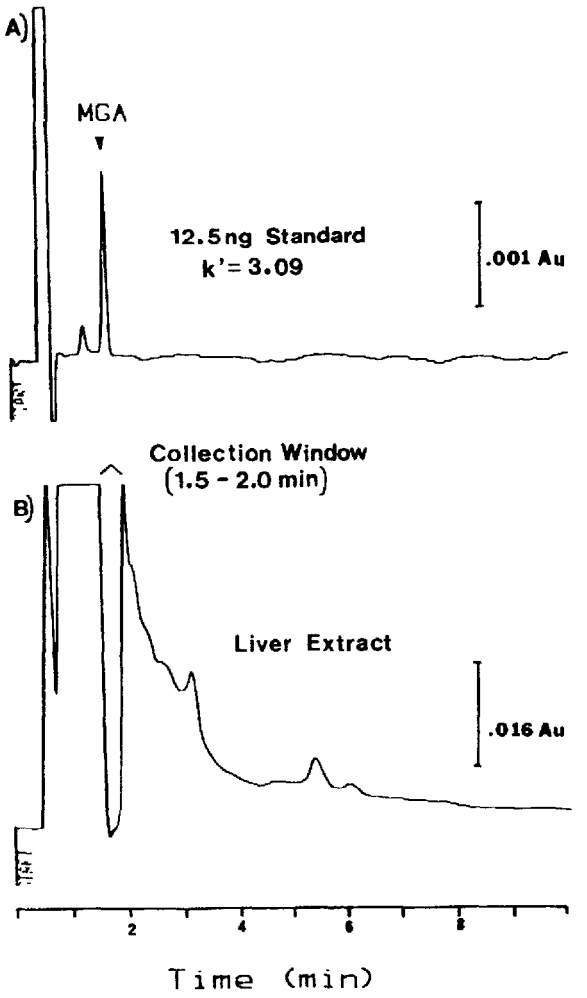


Fig. 3. HPLC profiles of an MGA standard (A) and liver extract (B) from the phenyl column (cf. Fig. 2) during the collection time. Detection was at 254 nm. The flow-rate (1.5 ml/min) and mobile phase composition (11% solvent A in B) were the same for chromatograms A and B. Eluent from column 1 was collected from 1.5 to 2.0 min onto the enrichment column (column 2).

The columns utilized in this system were as follows: column 1 was a 50 mm × 4.6 mm I.D., 3-μm Spherisorb phenyl, lot No. 23/163 (Keystone Scientific, State College, PA, U.S.A.), column 2, the enrichment column, was a DuPont, 12 mm × 4 mm I.D., 5-μm silica (Mac-Mod Analytical, Chadds Ford, PA, U.S.A.), and the final analytical column was a 100 mm × 4.6 mm I.D., 5-μm silica (Brownlee Labs., Santa Clara, CA, U.S.A.).

The retention time of MGA on the first column was verified daily by injecting 10–20 ng of analytical standard dissolved in cyclohexane. Actuation of the switching valves was then programmed accordingly to ensure quantitative collection of MGA from tissue extracts and standard solutions injected on the first column. The k' of MGA was typically 3.0 on the phenyl column while the average collection window was 0.5–0.6 min wide (Fig. 3).

TABLE I

COLUMN-SWITCHING TIMETABLE

Time (min)	Switching valve A	Switching valve B	Event
0	Position 2	Postion 2	Sample injection
1.5	Position 1		Begin collection of MGA onto column 2
2.0	Position 2		End collection, start integrator, begin separation on column 3
2.2		Position 1	Begin regeneration of column 1
6.2		Position 2	End regeneration of column 1, begin equilibration
10.2	Position 2	Position 2	Next injection

Mobile phases were composed of dichloromethane modified with 5% methanol and 0.1% water (solvent A) and hexane (solvent B). The mobile phase for the phenyl column was initially 9% solvent A in B. The mobile phase for column 3 was typically 14% solvent A in B. Flow-rates for columns 1 and 3 were 1.5 ml/min. The composition of the mobile phases was controlled by equilibrating the headspace of each solvent reservoir with the headspace of 125-ml Erlenmeyer flasks containing a small volume (10–20 ml) of dichloromethane and sealed with aluminum foil. The mobile phases and regeneration solvent were contained in capped reservoirs and sparged with helium for 2 min before priming the LC pumps. The regeneration solvent for the primary column was a 49:49:2 (v/v/v) mixture of methanol–dichloromethane–water. The flow-rate of the regeneration solvent was 2 ml/min for 4 min. Routing of the regeneration solvent to the phenyl column was automated with the second switching valve. Actuation of the regeneration–equilibration cycle began immediately after the start of analytical separation on column 3. The column switching events are described in Table I.

Quantification

MGA was quantified with external standards. Control tissue homogenates were spiked with 100 μl of ethyl acetate solutions containing MGA analytical standard. Extracts of these tissue homogenates were prepared for coupled-column determination using the three-phase liquid–liquid extraction procedure described above. The peak area of MGA was used to generate the calibration curves for quantitative analysis. For quantification of unknown liver tissues the levels ranged from 5 to 50 ppb and for muscle the range was from 2 to 20 ppb. The results of duplicate injections were averaged for establishing the calibration curves and assaying the incurred extracts. Calibration curves were established using linear least-squares regression analysis. The quantification of tissue extracts which were stored at $-20\,^{\circ}C$ for two months was also done to assess the stability of MGA in the extracts.

Determination of recovery

The recovery of MGA from the tissue homogenates in the liquid–liquid extraction procedure was determined by the comparison of peak areas obtained after

duplicate injections of standards and control liver tissue samples fortified with 10 and 50 ppb of MGA.

Preparation of HFAA derivative

The HFAA derivative of MGA was prepared after coupled-column HPLC purification of tissue extracts to produce the 3-heptafluorobutyryl enol ester of MGA (MGA-HFB). MGA was collected in the eluent after separation on the final silica analytical column. The eluent was then concentrated to dryness under nitrogen at 40°C. The residue was reconstituted in 100 μl of benzene and 10 μl of HFAA. After vortex-mixing the samples were heated at 70°C for 1 h and then concentrated to dryness at ambient temperature under a flow of nitrogen. The final residue was then re-dissolved in 10 μl of a 5% solution of ethyl acetate in isooctane and 3 μl were injected for GC–MS analysis.

Parameters for selected-ion monitoring (SIM)

The full-scan electron ionization (EI) mass spectrum for the HFB derivative of MGA is shown in Fig. 4. Accurate mass measurement by high-resolution MS was done for the HFB derivative. Measurements were made by the peak matching technique on a Kratos MS890MS hybrid mass spectrometer at a resolution of 15 000. The elemental compositions and exact mass measurements that were determined are given in Table II for the six ions chosen for SIM including the molecular ion (*m/z* 592) and five characteristic fragment ions (*m/z* 367, 381, 489, 517, and 533).

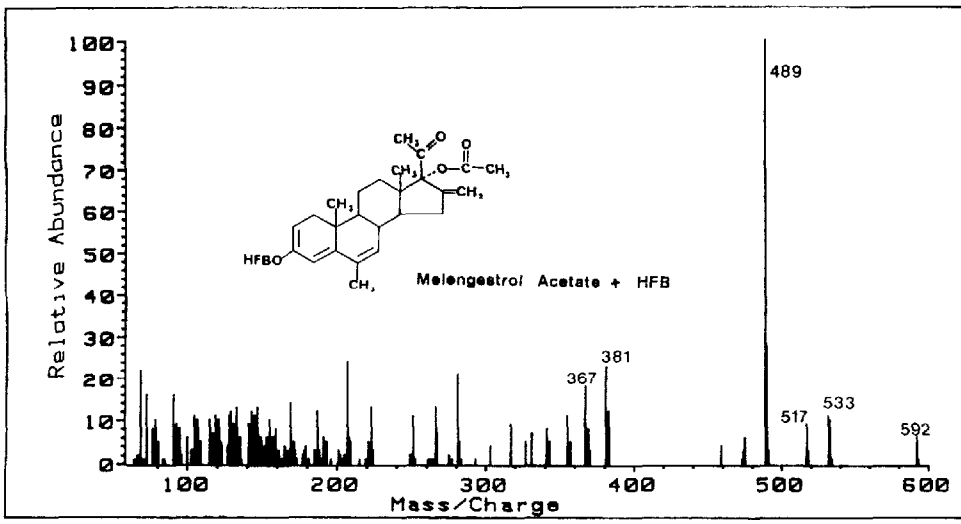


Fig. 4 Full-scan EI mass spectrum of MGA-HFB (40 ng). The ions chosen for selected-ion monitoring experiments are labeled.

TABLE II

HIGH-RESOLUTION MS ACCURATE MASS MEASUREMENT OF IONS MONITORED BY SIM FOR MGA-HFB

m/z	Measured mass	Calculated mass	Formula composition	Error (m.m.u.)
367	367 0565	367.0571	$C_{24}H_9F_2O_2$	−0.53
381	381.0703	381.0727	$C_{25}H_{11}F_2O_2$	−2.43
489	489.1672	489.1665	$C_{25}H_{24}F_7O_2$	0.81
517	517.1601	517.1614	$C_{26}H_{24}F_7O_3$	−1.29
533	533.1906	533.1927	$C_{27}H_{28}F_7O_3$	−2.09
592	592.2039	592.2060	$C_{29}H_{31}F_7O_5$	−2.09

GC–MS instrumentation

GC–MS analysis was done using a Hewlett-Packard (Avondale, PA, U.S.A.) gas chromatograph (Model 5890) interfaced to a mass-selective detector (MSD, Model 5970, Hewlett-Packard, Palo Alto, CA, U.S.A.). The capillary column was an HP Ultra-performance, 12 m × 0.2 mm I.D. fused-silica cross-linked methyl silicone with a film thickness of 0.33 μm. The injector temperature was maintained at 260°C and the interface temperature was 300°C. The initial oven temperature was 40°C followed by an immediate temperature program at 30°C/min to 150°C followed by a second temperature program at 6°C/min from 150 to 300°C. The final oven temperature was held for 5 min.

RESULTS AND DISCUSSION

Extraction

The three-phase liquid–liquid extraction of tissue homogenates provided a crude fractionation of the analyte from matrix components. The utility of this extraction procedure has been demonstrated for the determination of several anabolic substances which are under regulatory scrutiny including diethylstilbestrol (DES), estradiol, zeranol [16,17], and trenbolone [18]. While very lipophilic sample components partition into the upper hexane layer, these anabolic agents, including MGA, partition into the middle acetonitrile layer of intermediate polarity. Evaporation of this middle layer produced ca. 90 mg of an oily residue from 5 g of liver tissue. The residues were insoluble in methanol–water and acetonitrile–water mixtures that are most commonly employed in reversed-phase HPLC determination of steroids [19]. However, the residues were readily soluble in non-polar solvents, such as hexane, without further purification which made them compatible with a normal-phase HPLC system.

Chromatography

Giddings [15] has discussed coupled-column chromatography as one of many two-dimensional separation systems that can be employed for the analysis of complex samples. While the effectiveness of a single chromatographic system can

be expressed by its peak capacity, the total peak capacity of a coupled-column system is expressed as the product of the peak capacities of two or more columns in the system. The multiplicative property of a coupled-column system is applicable and fully realized when each column in the system achieves resolution through unique and independent separation mechanisms.

The most common applications of coupled-column liquid chromatography use precolumns operated in the enrichment–displacement mode for automated sample extraction with non-selective [9,20,21] or selective [22–25] stationary phases. Some reports have also demonstrated the use of two analytical columns with different selectivity to obtain two-dimensional LC separations [26,27].

In this work we investigated the possibility of using two analytical normal-phase columns with different selectivity and high efficiency for clean-up and separation of MGA from tissue extracts. To fully utilize the efficiency of a coupled-column system, stationary phases and their phase-volume ratios (surface area) must be chosen so that the eluent from the first analytical column is a weaker eluent for the second analytical column. Among the most common stationary phases the retention in the normal-phase mode will increase in the order: phenyl–silica < cyanopropyl–silica < diol–silica < silica [28]. Initially we investigated a coupled-column system comprised of a cyanopropyl column for the first separation and a silica column for the final separation. Almost the same eluent strength has to be used on both columns to elute MGA and the difference in selectivity was not sufficient to resolve MGA from tissue matrix components. A phenyl–silica combination gave less retention with the possibility of interactions between the conjugated MGA bonds and the aromatic phenyl groups of the stationary phase. A large difference in selectivity between the phenyl and silica columns was obtained as can be seen by comparing the chromatograms of Figs. 3B and 5.

The retention time of MGA on the first column was a critical parameter for the coupled-column method. To ensure the quantitative transfer of MGA onto the enrichment column the retention time must be reproducible. Several measures were taken to control retention in the normal-phase system. Retention was most affected on the phenyl column by polar matrix components which could not be eluted with the mobile phase. This necessitated the introduction of a regeneration cycle into the methodology. The composition of the regeneration solvent was designed to efficiently clear the column of retained matrix and allow for a practical equilibration time of the phenyl column. The latter was accomplished with the addition of water (2%) to the solvent which served to maintain the surface concentration of water during regeneration with a stronger eluent. The minimum time needed for equilibration was 4 min. The water content of the mobile phases was controlled by the addition of 0.1% water to solvent A.

Regeneration of the analytical silica column was necessary when the resolution of MGA from matrix components became difficult. Loss of resolution was accompanied by increased retention time of MGA. Many injections (300–400) of tissue extract were analyzed on the analytical silica column before regeneration became necessary. Regeneration of column 3 was achieved with 250 ml of a 50:50 solution of methanol–dichloromethane maintained at a flow-rate of 1.0 ml/min. Equili-

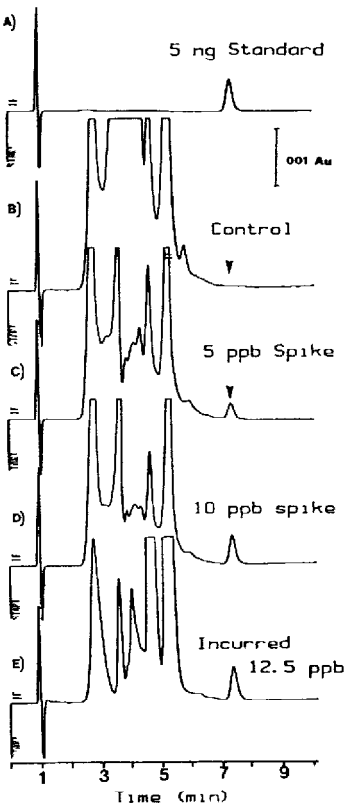


Fig. 5. HPLC–UV (287 nm) profiles of MGA standard (A) and liver extracts (B–E) from the final silica analytical column. The incurred liver level (E) resulted from the administration of 0.5 mg/day (seven days) of MGA. The chromatograms represent the equivalent of 0.5 g of liver tissue injected on-colum. The mobile phase compositions were 12 and 16% solvent A in B for columns 1 and 3, respectively.

bration of column 3 was achieved most quickly by first flushing the column with a 25% A in B mobile phase (50 ml) for 1 h followed by the mobile phase to be used for analysis of MGA (i.e. 14% A in B).

Band-broadening

The total peak-volume variance in a coupled-column system with enrichment between the two analytical columns can be expressed by the equation:

$$\sigma_{cc}^2 = \sigma_{inj}^2 + \left(\sigma_{c1}\frac{k'_e}{k'_c}\right)^2 + \sigma_{c3}^2 + \sigma_{det}^2$$

where σ_{cc} equals the total peak volume variance after coupled-column chromatography. The terms σ_{inj}^2, σ_{c1}^2, σ_{c3}^2, and σ_{det}^2 are the variance contributions of the injector, column 1, column 3 (without column-switching), and the detector, respectively. The ratio k'_e/k'_c represents the enrichment factor created during the fraction collection and elution with capacity factors of k'_c and k'_e, respectively [6]. If extra-column contributions are regarded as minor contributions the ratio

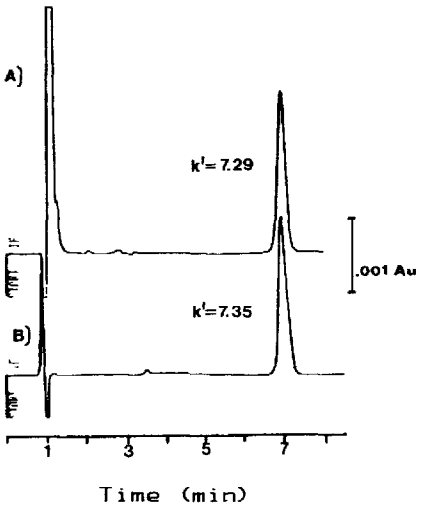


Fig. 6. HPLC-UV (287 nm) profiles for an MGA standard (21 ng) on the final silica analytical column without column switching (A) and with column switching (B). The flow-rate (1.5 ml/min) and mobile phase composition (14% solvent A in B) are the same for both HPLC profiles.

of the peak widths on the last column with and without coupled-column separation can be expressed as:

$$\frac{\sigma_{cc}}{\sigma_{c3}} = \sqrt{\frac{\left(\sigma_{c1}\frac{k'_e}{k'_c}\right)^2 + \sigma_{c3}^2}{\sigma_{c3}^2}}$$

which gave a ratio of 1.017 with $\sigma_{c1}=0.078$, $k'_e/k'_c=1/3$, and $\sigma_{c3}=0.140$. This value corresponds well with observed peak widths as can be seen in Fig. 6 and corresponds to a loss of efficiency from 47 748 and 43 938 plates/m.

Recovery and precision

To determine the recovery of MGA by the liquid–liquid extraction two levels of MGA (10 and 50 ppb) were spiked into control liver homogenates. The average recovery at both levels was 85.6%. The results of duplicate injections were averaged for 23 extracts of each level. The inter-assay precision has been determined and is summarized in Table III.

The incurred liver tissues were quantified for MGA by on-line coupled-column HPLC using external standards on four consecutive days. Calibration curves were obtained for each day of the study. The last set of extracts which were analyzed had been stored at −20°C for two months including the extracts used for the calibration curve. The average levels were determined to be 12.5, 18.3, and 31.6 ppb for the animals that were administered 0.5, 1.5, and 3.0 mg of MGA, respectively (σ=1.27, 1.00, and 2.17, respectively, n=4). Correlation coefficients for the calibration curves were 0.999 (σ = 0.001) with the levels of MGA ranging from 5 to 50 ppb (i.e. 5, 10, 20, and 50 ppb). The average standard error of estimate for the calibration curves was 0.494 (σ=0.309, n=4).

TABLE III

INTER-ASSAY PRECISION AND RECOVERY

Level	$\bar{x}$ (%)	σ	C.V. (%)	n
10 ppb	76.1	5.65	7.42	8
	83.5	1.93	2.31	3
	90.1	1.46	1.62	4
	92.7	3.74	4.03	8
	85.3	8.40	9.84	23
50 ppb	84.3	2.09	2.48	8
	85.2	1.90	2.23	8
	88.5	2.56	2.89	7
	85.9	2.76	3.22	23

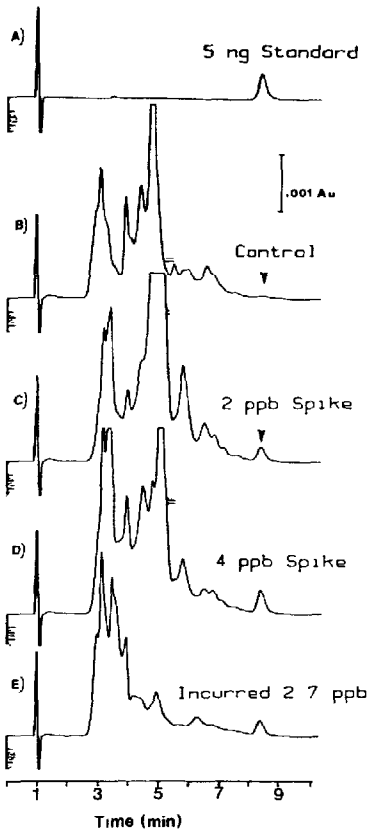


Fig. 7. HPLC–UV (287 nm) profiles of MGA standard (A) and muscle extracts (B–E) from the final silica analytical column. The incurred muscle level shown in chromatogram E resulted from the administration of 3.0 mg/day (seven days) of MGA. These chromatograms represent the equivalent of 1.25 g of muscle tissue injected on-column. The mobile phase compositions were 10 and 14% solvent A in B for columns 1 and 3, respectively.

Glaser et al. [29] have described a method for defining the LOD of an analytical technique. Based on these criteria the calculated LOD for the coupled-column method for MGA in liver tissue is 3.0 ppb. The LOD required for this work was 5 ppb. This capability is demonstrated in Fig. 5C with the HPLC profile of a 5-ppb liver spike. Muscle extracts were considerably 'cleaner' than those of the liver. This allowed the injection of more concentrated samples. The quantification of MGA in muscle extracts was achieved at the 2-ppb level and is shown in Fig. 7. The chromatograms of control liver and muscle extracts (Figs. 6B and 7B) show that there were no significant interferences from endogenous components on the silica analytical column at the wavelength used (287 nm). Chromatograms from kidney extracts (Fig. 8), on the other hand, demonstrate some interference with MGA on the final silica column. This interference precluded accurate quantification of MGA at the 5-ppb level in the incurred kiney extracts although HPLC screening of all incurred kidney samples indicated the presence of MGA.

MGA was also quantified in the incurred fat tissues (visceral and perirenal) from the dosing study. This work was conducted by the Midwestern USDA-FSIS laboratory using the regulatory method for the determination MGA [3]. The average levels of MGA in the fat tissues were 35, 59, and 97 ppb for the animals that were dosed with 0.5, 1.5, and 3.0 mg of MGA, respectively. The average relative ratio of MGA levels in liver/fat tissues was 33.1% [30]. This corroborates with the liver/fat tissue ratios of MGA residue levels which were determined by

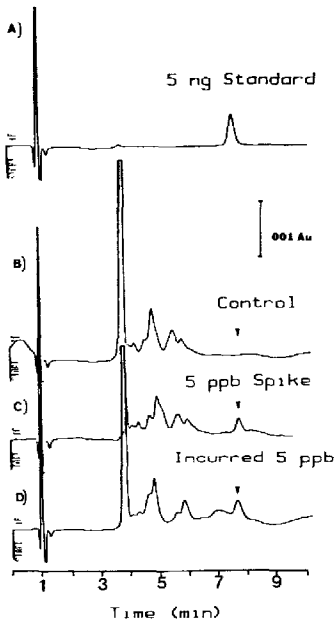


Fig. 8. HPLC-UV (287 nm) profiles of MGA standard (A) and kidney extracts (B–D) from the final silica analytical column. The incurred kidney level shown resulted from the administration of 1.5 mg/day (seven days) of MGA. These chromatograms represent the equivalent of 0.5 g of kidney tissue injected on-column. The mobile phase compositions were 9 and 14% solvent A in B for columns 1 and 3, respectively.

Krzeminski et al. [31] in conjunction with a radiolabeled study. Using the regulatory method they concluded that fat tissue contained the highest percentage (86%) of intact, parent MGA and the liver contained less (29%) resulting in a relative ratio (liver/fat) of 33.7%.

Method ruggedness

Approximatelfy 1000 injections of crude tissue extract (the equivalent of 10 g of oily liver extract) were made on the phenyl column. Its chromatographic capability remained constant although the retention characteristics changed slowly with an increased number of tissue extract injections. This was compensated for by gradually increasing the content of solvent A in B from 9 to 12% to maintain a capacity factor of ca. 3 for MGA on the phenyl column.

The capacity factor for MGA on the phenyl column was found to vary between packing materials from two different lots. Phenyl packing from lot No. 23/163

TABLE IV

BLIND-ANALYSIS STUDY: RECOVERY OF MGA FROM BOVINE LIVER (SPIKED AND INCURRED TISSUES)

Level added (ppb)	Concentration found (ppb)	Error (%)
0 (Control)	N.D. (<3 ppb)[a]	0
0 (Control)	N.D. (<3 ppb)	0
0 (Control)	N.D. (<3 ppb)	0
10	9.7	−3.0
10	8.7	−13.0
10	10.1	+1 0
20	18.1	−9.5
20	18.3	−8.5
20	18.6	−7.0
40	36.5	−8.8
40	37.1	−7.3
40	38.5	−3.8
		$\bar{x}$= −6.7
		σ= 4.1
		n= 9
Incurred level (ppb)	**Concentration found (ppb)**	**Error (%)**
12.5	13.1	+4.8
12.5	14.9	+19.2
12.5	12.0	−4.0
18.3	19 2	+5.0
18 3	18.1	−1.1
18.3	20.1	+9.8
		$\bar{x}$= 5.6
		σ= 8.3
		n= 6

[a]N D. = not detected; limit of detection is 3 ppb.

TABLE V

BLIND-ANALYSIS STUDY: RECOVERY OF MGA FROM BOVINE MUSCLE (SPIKED AND INCURRED TISSUES)

Level added (ppb)	Concentration found (ppb)	Error (%)
0 (Control)	N.D. (<2 ppb)[a]	0
0 (Control)	N.D. (<2 ppb)	0
4	3.4	−15.0
4	3.4	−15.0
10	9.9	−1.0
10	9.6	−4.0
20	18.9	−5.5
20	19.8	−1.0
		$\bar{x}$= −6.9
		σ= 6.5
		n= 6
Incurred level (ppb)	**Concentration found (ppb)**	**Error (%)**
2.7	2.8	+3.7
2.7	3.0	+11.1
		$\bar{x}$= 7.4

[a]N.D. = not detected; limit of detection is estimated to be less than 2 ppb.

TABLE VI

ION PEAK-AREA RATIO STATISTICS FOR MGA-HFB (STANDARDS AND RESIDUES IN BOVINE TISSUE EXTRACTS)

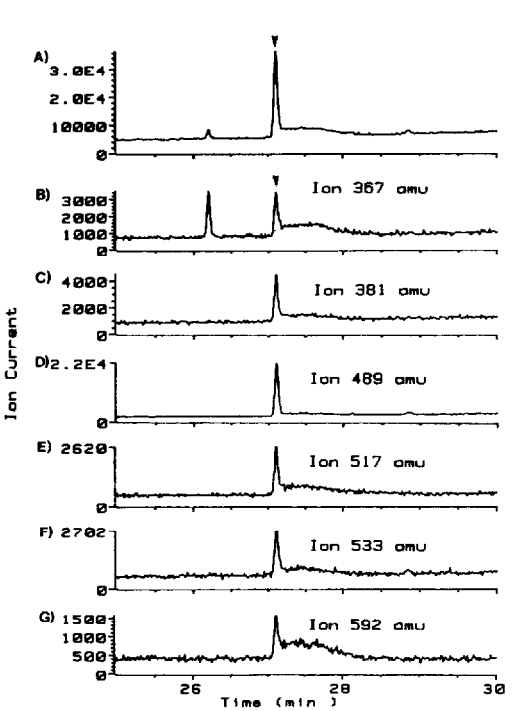
The values in parentheses are the number of samples (n).

	592/489	533/489	517/489	381/489	367/489
MGA-HFB standard[a]	0.053±0.004 (5)	0.106±0.005 (5)	0.104±0.008 (5)	0 187±0.019 (5)	0.136±0.010 (5)
Liver	0.053±0.003 (15)	0.114±0.006 (15)	0.107±0 005 (15)	0.169±0.007 (15)	0.129±0.006 (15)
Muscle	0.050±0.003 (8)	0.108±0.008 (8)	0.105±0 003 (8)	0.188±0.011 (8)	0.146±0 016 (8)

[a]Standards of 1.5 ng were used for these determinations

(Spherisorb, Keystone Scientific) provided the best selectivity for MGA in the normal-phase mode.

A blind-analysis study was conducted as part of an inter-laboratory validation study to assess the utility of the coupled-column method for regulatory use. The objectives of the study were to demonstrate the quantification of MGA in bovine liver and muscle tissue extracts using the coupled-column HPLC methodology and to confirm the presence of MGA in the purified extracts using SIM capillary GC–MS. Three sample sets of liver tissue (six samples per set) and two sample sets of muscle tissue (five samples per set) were quantified by coupled-column HPLC. Each set of tissues included one control and incurred liver or muscle tissues which had been quantified prior to the study. The results of the quantitative



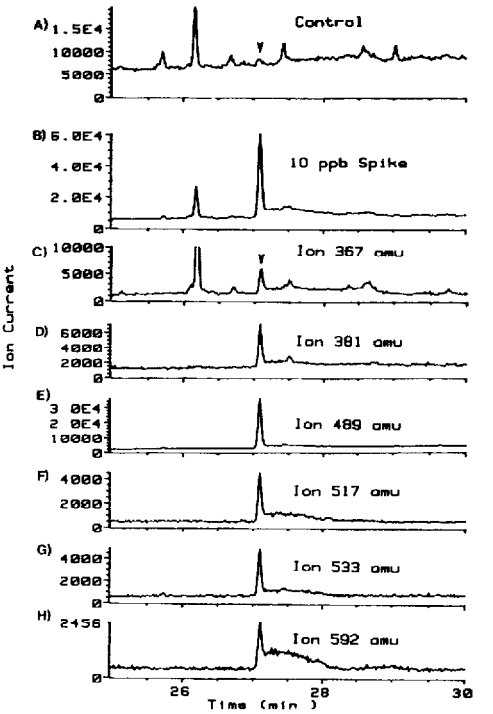


Fig. 9. Total selected-ion current capillary GC–MS profiles (A) and selected-ion current profiles (B–G) of a 1 5-ng standard of MGA-HFB.

Fig. 10. Total selected-ion current capillary GC–MS profiles of a control liver extract (A) and a 10-ppb liver spike (B). The selected-ion current profiles (C–H) of the liver spike are shown for the six ions chosen for the confirmation of MGA-HFB.

portion of the study are given in Tables IV and V for liver and muscle tissues, respectively. There was a significant difference between the level of MGA which was added and the concentration which was found ($p < 0.01$). The mean of concentrations found were 6.8% lower than the concentrations added.

The blind-analysis study was conducted for three consecutive days. The average retention times of MGA on the final silica column were 6.97 ± 0.12, 7.59 ± 0.08, and 6.85 ± 0.049 min ($n = 22$, 39, and 30, respectively). The retention time of MGA on the phenyl column was verified at the beginning of each working day. During this study and throughout the development of the coupled-column method the retention time of MGA on the phenyl column was very reproducible for any given day and mobile phase composition.

Confirmation of MGA

MGA is reported to be unstable at high temperatures [1]. Several attempts were made to assay MGA directly by capillary GC–MS for this study, but these efforts were unsuccessful. HFB enol ether derivatives of 3-oxo-Δ-steroids have been reported earlier using HFBA and benzene [32,33]. The HFB derivative was

used, therefore, because it is thermally stable and provides higher-mass ions which results in an increased SIM specificity.

The criteria used for the confirmation of MGA-HFB in tissue extracts included SIM detection of the molecular ion (m/z 592) with a signal-to-noise ratio of at least 3. In addition, the five ion ratios established from the six selected-ion peak areas were to be within 15% of the ratios of a standard analyzed under the same conditions. Table VI summarizes the ion ratio statistics for the confimatory portion of the blind-analysis study described above. MGA-HFB was confirmed from purified muscle and liver extracts which had been purified by coupled-column HPLC as described above. The level of MGA in these samples ranged from 4 to 25 ng.

The total selected-ion current chromatogram and selected-ion current profiles of an MGA-HFB standard are shown in Fig. 9. The corresponding SIM chromatograms for purified liver extracts from the study (control and 10-ppb spike) are shown in Fig. 10.

CONCLUSIONS

A coupled-column HPLC method has been described for the automated sample purification, on-line screening, and quantification with external standards of MGA in bovine tissue extracts. A combination of phenyl and silica analytical columns operated in the normal-phase mode provided a large difference in selectivity which allowed for the efficient isolation of MGA. Tissue extracts, purified by the coupled-column method, were suitable for GC–MS confirmation. MGA was confirmed as its HFB derivative in tissue extracts following purification by the coupled-column method.

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