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Determination of tannic acid and its phenolic metabolites in biological fluids by high-performance liquid chromatography

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ABSTRACT

A method for the identification and determination of tannic acid and its phenolic metabolites in biological fluids by high-performance liquid chromatography was developed. Tannic acid and four phenolic compounds, namely gallic acid, pyrogallol, 4-O-methylgallic acid and ellagic acid, were successfully extracted from the biological fluids by using ethyl acetate at acidic conditions. Gallic acid, pyrogallol and 4-O-methylgallic acid were found in the sheep urine, gallic acid, 4-O-methylgallic acid and ellagic acid in plasma, and gallic acid and ellagic acid in abomasal fluid after abomasal dosing of tannic acid. Tannic acid was found in the plasma apart from the abomasal fluid into which it was administered. The concentrations of tannic acid, gallic acid, pyrogallol, 4-O-methylgallic acid and ellagic acid in plasma, abomasal fluid and urine were measured. This method could be applied to measurement of other hydrolysable tannins and their phenolic metabolites in bioligical materials.

INTRODUCTION

Tannins found in plants are polyphenolic polymers with molecular masses ranging from 500 to 3000. Tannins are generally divided into hydrolysable and condensed tannins. They are widely used in the brewery industry and leather tanning industry because of their protein-precipitating property [1,2]. However, high contents of hydrolysable tannins in certain species of plants, such as oak (*Quercus spp.*) [3] and yellow-wood (*Terminalia oblongata*), can cause poisoning in animals if large amounts of these plants are ingested [4,5]. In order to study the pathogenesis of hydrolysable tannin toxicity, a reliable method for determining tannic acid (TA) and its phenolic metabolites in biological materials needs to be developed.

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Various methods for determining TA and phenols in biological materials have been reported using spectrophotometry [6,7], paper chromatography [8,9] and thin-layer chromatography (TLC) [10], but these methods either are non-specific or lack sensitivity. Gas chromatography (GC) offers high sensitivity for a wide range of phenolic compounds; however, it generally requires a derivatisation step before detection [11,12]. Although the measurement of TA using high-performance liquid chromatography (HPLC) has previously been reported [2,13,14], TA was measured in standard solutions instead of biological materials.

Due to the complex matrices of biological fluids, especially urine, it is necessary to separate TA and other phenolic compounds from the interfering substances before injection into the HPLC column. Methods for the determination of TA and its phenolic metabolites in plasma, abomasal fluid and urine obtained from a sheep given a sublethal dose of TA have been developed and are reported.

EXPERIMENTAL

Chemicals

All chemicals are of analytical grade unless specified. TA (L.R. grade) was purchased from Ajax Chemicals (Sydney, Australia), gallic acid (GA), pyrogallol (PYR), phenol, catechol (CAT), resorcinol (RES) and ellagic acid (EA) from Sigma (St. Louis, MO, USA), 4-O-methylgallic acid (4OMGA) from Spectrum Chemical (Gardena, CA, USA) and protocatechuic acid (PA) from Aldrich (Milwaukee, WI, USA). Benzoic acid (BA), 4-hydroxybenzoic acid (4HBA), phosphoric acid and ethyl acetate were obtained from British Drug House (BDH Australia, Kilsyth, Australia). Methanol (HPLC grade) was obtained from Mallinckrodt (Mallinckrodt Australia, a Division of Rhone-Poulenc, Clayton, Australia). Type I pure water (18 MΩcm) was obtained by passing reverse-osmosis water through a Millipore Q water purification system (Millipore, Milford, MA, USA). All stock solutions were prepared in pure methanol.

Collection of samples

Blood samples were collected into heparinized blood containers (Johns Professional Products, Cheltenham, Australia) from the external jugular vein of the sheep. Plasma samples were immediately obtained by centrifugation of the blood at 1800 g for 10 min. Abomasal fluid was obtained via an abomasal fistula which was surgically inserted at least two weeks before dosing [15], while urine was obtained via a urethral catheter as previously described by Zhu and Filippich [16]. All samples were stored at −20°C and before being analysed defrosted at room temperature, mixed and centrifuged at 1800 *g* for 10 min. All samples were assayed in duplicate.

Extraction of TA, GA, PYR and 4OMGA from plasma

To determine the optimal recovery of TA, GA, PYR and 4OMGA from plasma, addition of different amounts of sulphuric acid with or without hydrolysis were evaluated (see Table 1). The sheep plasma used in this recovery study was obtained from the blood collected before TA was dosed to the animal.

For TA extraction, 1 ml of plasma was spiked with 20 μl of TA (9.384 g/l) and 20 μl of PA (1.074 g/l) (internal standard), and an equal volume of 1 *M*, 5 *M* or concentrated sulphuric acid was added in Kimax glass culture tubes (125 mm × 16 mm) with PTFE-coated screw caps (Kimble, Vineland, NY, USA). Each tube was thoroughly mixed on a vortex mixer (Scientific Industries, Springfield, MA, USA) before extraction with ethyl acetate. In another set of tubes, 1 ml of plasma was spiked with PA and TA, and, after the addition of sulphuric acid as above, the mixture was hydrolysed in a boiling water bath for 20 min and then cooled with running tap water. Both sets of spiked samples were extracted with two 4-ml portions of ethyl acetate by mixing on a rotator mixer (Analite, Melbourne, Australia) for 30 min. After centrifugation at 1800 *g* for 15 min, the organic phase was pipetted to another glass tube. The combined ethyl acetate extracts were dried in an aluminium block heater (Thermoline, Melbourne, Australia) at about 40°C

with a stream of air. The residue was dissolved in 2 ml of methanol and a 20-μl aliquot was injected onto the HPLC system for analysis. For recovery of PYR, GA and 4OMGA from plasma, a 60-μl mixture of the three compounds at concentrations of 3.275, 0.3244 and 0.364 g/l, respectively, and 20 μl of PA were added to 1 ml of plasma. The same method as described for TA extraction was used.

Hydrolysis study of urine samples

Urine samples collected from one sheep over a 12-h period following abomasal administration of TA were used for the hydrolysis study. A 1-ml 20:1 diluted urine sample, 20 μl of PA and 1 ml of 1 *M* sulphuric acid were added to six glass tubes. Two tubes were hydrolysed in a boiling water bath for 20 min, two for 40 min, and then immediately cooled wih running tap water. The four hydrolysed samples and the two samples without hydrolysis were then extracted with one 8-ml portion of ethyl acetate for 30 min. After centrifugation at 1800 *g* for 10 min, the organic fraction was separated and dried as described above. The residue was dissolved in 2 ml of methanol and a 20-μl aliquot was injected onto the HPLC system for analysis. At the same time, the control urine collected before dosing of TA was spiked with 20 μl of PA and 60 μl of a standard mixture of PYR, GA and 4OMGA, and treated as described for the test samples. Concentrations of PA, PYR, GA and 4OMGA were the same as those used in the extraction from plasma described above.

Preparation of plasma samples

After the initial study on the extraction method using various acid concentrations, plasma samples (1–3 ml) were extracted with ethyl acetate (2 × 4 ml) after the addition of an equal volume of 1 *M* sulphuric acid and 20 μl of PA without hydrolysis.

Preparation of abomasal fluid

Abomasal fluid was diluted 2–40 times with water before extraction as described for plasma.

Preparations of urine samples

After the hydrolysis study of spiked and test urine samples, urine was diluted 2–50 times and extracted with ethyl acetate (1 × 8 ml) after the addition of an equal volume of 1 *M* sulphuric acid and 20 μl of PA and 20 min hydrolysis.

Calibration and quantitation

GA, PYR, 4OMGA and EA concentrations were calculated using the analyte/internal standard (PA) peak-height ratios and calibrated from the standard curves and dilution factors, while TA was calculated using the TA/PA peak-area ratio. The peak area of TA was calculated by subtracting the peak area of the internal standard (PA) and the total peak area of the relative control sample and GA, 4OMGA and/or EA, if applicable, from the total peak area of the test sample.

High-performance liquid chromatography

Samples were analysed on a HPLC system with an LKB 2150 dual-pump delivery system, an LKB 2152 HPLC controller (LKB, Bromma, Sweden), an ICI AS2000 HPLC automatic injector (ICI Instruments, Melbourne, Australia) and a Waters 490E programmable multi-wavelength UV–VIS detector (Millipore). The separation was achieved by using a Waters μBondapak reversed-phase C_{18} column (10 μm particle size; 300 mm × 4.6 mm I.D.) (Millipore) and a gradient elution with 0.025% phosphoric acid in water (A) and 0.025% phosphoric acid in methanol (B) at a flow-rate of 1 ml/min. The chromatogram was recorded and integrated by an IBM PC/AT-based computer with Delta chromatography data software (Digital Solutions, Brisbane, Australia). The gradient of elution was as follows: 10% B gradually increased to 64% B at 18 min, to 100% B at 20 min and returned to 10% B at 22 min. The eluent was monitored simultaneously at wavelengths of 254, 260 and 280 nm for maximum sensitivity. A Phenomenex IB-SIL reversed-phase C_8 column (5 μm particle size; 250 mm × 4.6 mm I.D.) (Phenomenex, Torrance, CA, USA) was also used to confirm that the metabolites found in sheep plasma, urine or abomasal fluid are present.

RESULTS AND DISCUSSION

TA is a mixture of polygalloylglucoses or polygalloylquinic acids depending on the plant source from which TA was extracted [2]. TA extracted from Chinese gall nuts (*Rhus semialata*) consists mainly of penta- to undecagalloylglucoses with depside galloyl group(s) randomly distributed at the C-2, C-3 and C-4 position(s) on a penta-O-galloyl-β-D-glucose core as determined by Nishizawa *et al.* [17]. There are also small amounts of GA and its oligomers (digallic acid and trigallic acid) in the commercial TA [14,18]. TA used in this study consists of several galloylglucoses, a small amount of GA and trace of EA as revealed by HPLC (Fig. 1A). Contents of GA and EA in the TA were found to be 3.36 and 0.17%, respectively. EA has not been found in commercial TA by other workers. It is possible that the Ajax TA was obtained from a different source than that used by other workers in their studies. Individual polygalloylglucose, digallic acid and trigallic acid were not determined because the standard materials were not available.

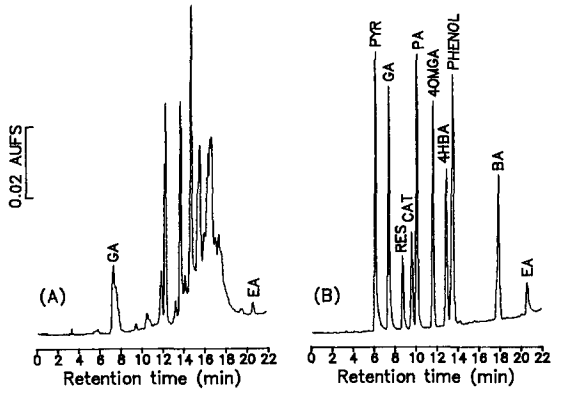

Fig. 1. Chromatograms of (A) standard tannic acid showing multiple peaks including gallic acid and ellagic acid and (B) a standard solution of ten phenolic compounds on a μBondapak C_{18} reversed-phase column. Mobile phase: (A) water containing 0.025% phosphoric acid; (B) methanol containing 0.025% phosphoric acid; gradient: 10% B to 64% B at 18 min, to 100% at 20 min, returned to 10% at 22 min; Flow-rate: 1 ml/min; Detection 254, 260 and 280 nm. Peaks: TA = tannic acid; PYR = pyrogallol; GA = gallic acid; RES = resorcinol; CAT = catechol; PA = protocatechuic acid; 4OMGA = 4-O-methylgallic acid; 4HBA = 4-hydroxybenzoic acid; BA = benzoic acid; EA = ellagic acid.

A baseline separation (Fig. 1B) of ten standard phenolic compounds, namely PYR, GA, CAT, RES, PA, 4OMGA, phenol, 4HBA, BA and EA, was obtained by using a C_{18} reversed-phase column and gradient elution with the mobile phase consisting of acidic water and methanol.

Extraction recoveries of TA, GA, PYR and 4OMGA from the spiked plasma under various acid concentrations and hydrolysis times are shown in Table I. For TA extraction, addition of 1 *M* sulphuric acid without hydrolysis gave maximal recovery and minimal degradation, while addition of 5 *M* and concentrated sulphuric acid with or without hydrolysis and hydrolysis with 1 *M* acid resulted in low recoveries of TA and degradation of TA to GA and other low-molecular-mass components as observed from the HPLC profiles (Fig. 2). The polygalloylglucose moiety is readily decomposed to GA, digallic acid and trigallic acid by acid hydrolysis [18].

HPLC profiles of plasma samples spiked with GA, PYR and 4OMGA under various conditions are shown in Fig. 3. GA, PYR and 4OMGA appear to be stable in plasma with or without hydrolysis using 1 *M* sulphuric acid; however, decomposition was observed in plasma after hydrolysis with 5 *M* or concentrated sulphuric acid and, subsequently, poor recoveries were obtained. Based on these findings, extraction of TA and phenolic compounds from plasma and abomasal fluid were carried out with 1 *M* sulphuric acid without hydrolysis.

Phenolic compounds are mostly excreted as conjugates in urine [19]. In order to measure GA, PYR and 4OMGA, urine should be hydrolysed to release these compounds from their conjugated froms before extraction with ethyl acetate. GA, 4OMGA and PYR in particular are not stable under highly acidic conditions as shown in the study of spiked plasma samples described above. The optimal conditions of hydrolysis without degradation of these phenolic compounds but with maximal release of phenolics from conjugated froms were explored. The results of various hydrolysis times of sheep urine after abomasal dosing of TA and control urine samples spiked with a standard mixture of GA, PYR and 4OM-

TABLE I

EXTRACTION RECOVERIES OF TA (46.92 mg/l), PYR (49.12 mg/l), GA (4.87 mg/l) AND 4OMGA (5.46 mg/l) FROM SPIKED PLASMA UNDER VARIOUS CONDITIONS

Sulphuric acid concentration[a] (*M*)	Hydrolysis time (min)	Extraction recovery (%)			
		TA	PYR	GA	4OMGA
1	0	91.94	68.61	95.79	103.02
	20	91-35	59.06	103.60	107.33
5	0	65.80	40.82	93.94	86.45
	20	67.91	21.47	99.28	84.62
Concentrated	0	60.07	72.37	78.62	90.29
	20	64.40	8.43	105.76	87.64

[a] Equal volume of sulphuric acid and plasma.

GA are shown in Fig. 4. It was found that the optimal duration of hydrolysis was 20 min with 1 *M* sulphuric acid. PYR was the only phenolic substance excreted in the conjugated form as its concentration was increased after hydrolysis, while GA and 4OMGA concentrations remained constant with or without hydrolysis.

Three phenolic metabolites, GA, PYR and 4OMGA, were found in the urine, GA, 4OMGA, and EA in the plasma, while only GA and EA, thought to be breakdown products of TA, in the abomasal fluid of sheep after abomasal dosing of TA. The HPLC profiles of control and test samples are shown in Fig. 5. These metabolites were

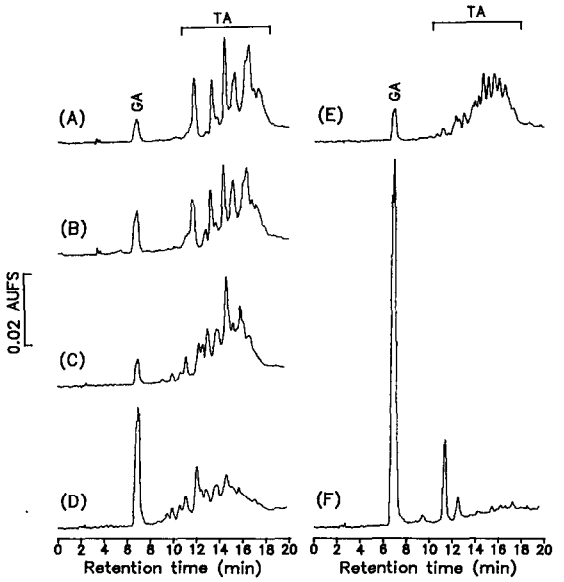

Fig. 2. Chromatograms of plasma samples spiked with standard TA extracted under various conditions (equal volumes of sulphuric acid were added). (A) 1 *M* sulphuric acid without hydrolysis; (B) 1 *M* sulphuric acid with 20 min hydrolysis; (C) 5 *M* sulphuric acid without hydrolysis; (D) 5 *M* sulphuric acid with 20 min hydrolysis; (E) concentrated sulphuric acid without hydrolysis; (F) concentrated sulphuric acid with 20 min hydrolysis. Refer to Fig. 1 for HPLC conditions and abbreviations.

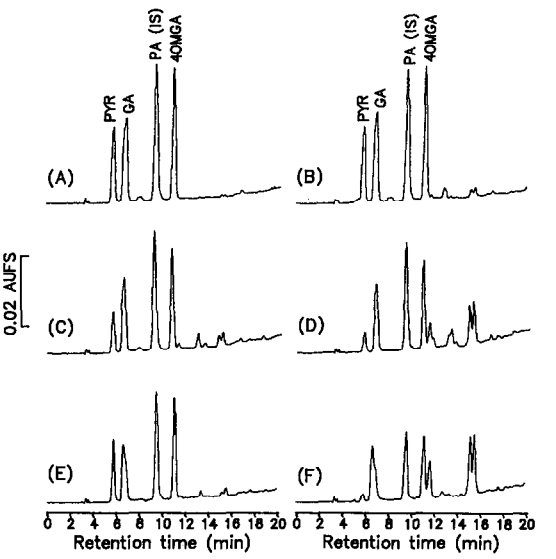

Fig. 3. Chromatograms of plasma samples spiked with a standard mixture of PYR, GA and 4OMGA extracted under various conditions (equal volumes of sulphuric acid were added). (A) 1 *M* sulphuric acid without hydrolysis; (B) 1 *M* sulphuric acid with 20 min hydrolysis; (C) 5 *M* sulphuric acid without hydrolysis; (D) 5 *M* sulphuric acid with 20 min hydrolysis; (E) concentrated sulphuric acid without hydrolysis; (F) concentrated sulphuric acid with 20 min hydrolysis. Refer to Fig. 1 for HPLC conditions and abbreviations.

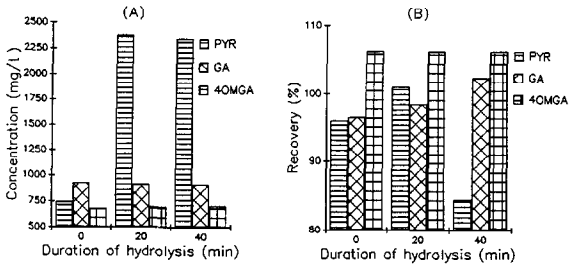

Fig. 4. (A) GA, PYR and 4OMGA concentrations found in the sheep urine collected over a 12-h period following abomasal dosing of TA at 1.0 g/kg body weight and (B) extraction recoveries of spiked urine samples at various times of hydrolysis. Refer to Fig. 1 for abbreviations.

confirmed by their retention times on a different column (reversed-phase C_8) using the same mobile phase and gradient as on the C_{18} column.

Recovery and precision of the extraction method were satisfactory. The recoveries and coefficients of variation in the determination of TA, GA, PYR, 4OMGA and EA from spiked plasma, abomasal fluid and urine samples are shown in Table II, III and IV, respectively. It was found that the recoveries of GA, 4OMGA and EA were over 85% from all samples examined. Recoveries of TA and PYR from plasma and abomasal fluid were lower than those of GA and 4OMGA, but they were consistent. Lower recoveries of TA and PYR from plasma and abomasal fluid could be caused by binding of TA and PYR to protein in plasma and abomasal fluid [20].

TA and its metabolites, namely GA, PYR, 4OMGA and EA in plasma, abomasal fluid and urine of sheep after abomasal dosing of TA at 1.0 g/kg body weight, were measured and the results are shown in Fig. 6. TA and its hydrolysis product, GA, were not detected in the abomasum 24 h after dosing of TA, while TA was still detectable in the plasma 48 h postdosing. This suggests that TA could be absorbed from both abomasum and

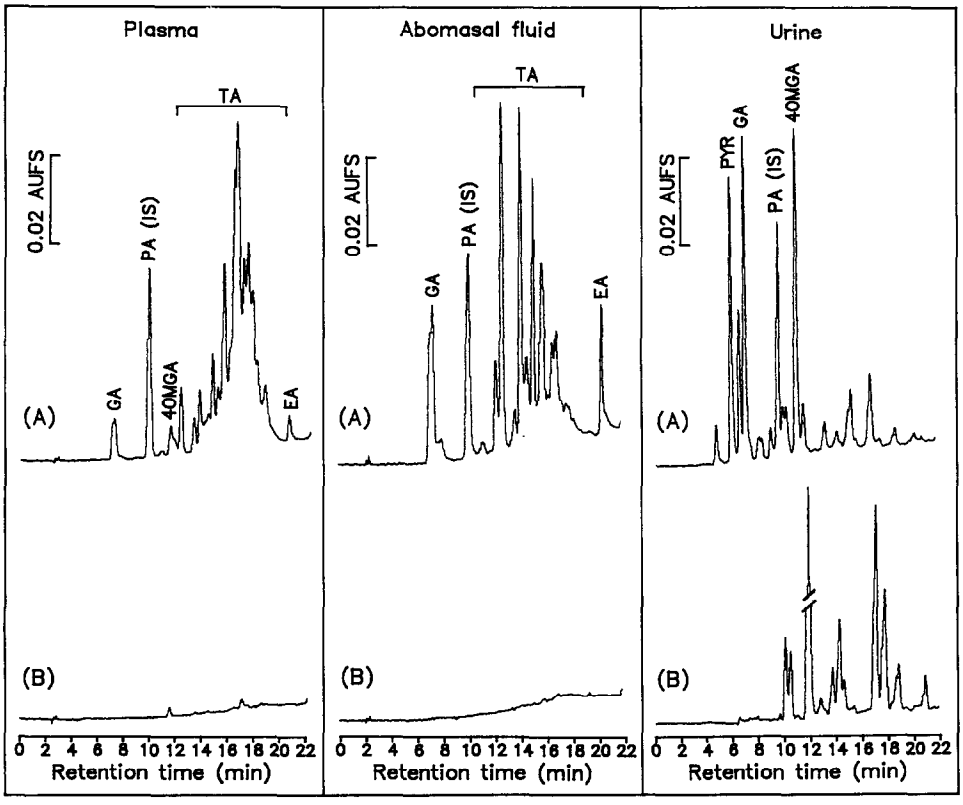

Fig. 5. Chromatograms of sheep plasma, abomasal fluid and urine extracts (A) after and (B) before abomasal dosing of 1.0 g TA per kg body weight. Refer to Fig. 1 for HPLC conditions and abbreviations.

TABLE II

RECOVERIES AND COEFFICIENTS OF VARIATION FOR THE DETERMINATION OF TA, GA, PYR, 4OMGA AND EA IN 1 ml OF SPIKED PLASMA

Compound	Amount added (μg)	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)	
			Intra-assay	Inter-assay
TA	187.68	87.96 ± 4.17 ($n=11$)	3.60 ($n=4$)	3.79 ($n=5$)
PYR	196.48	67.89 ± 6.84 ($n=10$)	3.35 ($n=4$)	11.23 ($n=4$)
GA	19.46	94.47 ± 4.11 ($n=12$)	3.79 ($n=4$)	3.06 ($n=5$)
4OMGA	21.84	95.11 ± 4.73 ($n=12$)	3.50 ($n=4$)	4.81 ($n=5$)
EA	5.89	108.60 ± 6.69 ($n=11$)	8.22 ($n=5$)	3.71 ($n=4$)

TABLE III

RECOVERIES AND COEFFICIENTS OF VARIATION FOR THE DETERMINATION OF TA, GA, PYR, 4OMGA AND EA IN 1 ml OF SPIKED ABOMASAL FLUID

Compound	Amount added (μg)	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)	
			Intra-assay	Inter-assay
TA	187.68	82.32 ± 7.65 ($n=10$)	8.04 ($n=4$)	7.00 ($n=4$)
PYR	196.48	71.29 ± 7.69 ($n=8$)	5.95 ($n=4$)	12.57 ($n=4$)
GA	19.46	88.34 ± 3.62 ($n=10$)	5.45 ($n=4$)	2.50 ($n=4$)
4OMGA	21.84	86.27 ± 8.50 ($n=10$)	5.11 ($n=4$)	9.72 ($n=4$)
EA	5.89	110.33 ± 8.77 ($n=10$)	7.76 ($n=5$)	7.17 ($n=4$)

TABLE IV

RECOVERIES AND COEFFICIENTS OF VARIATION FOR THE DETERMINATION OF GA, PYR AND 4OMGA IN 1 ml OF SPIKED URINE

Compound	Amount added (μg)	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)	
			Intra-assay	Inter-assay
PYR	196.48	98.20 ± 14.65 ($n=13$)	1.91 ($n=5$)	14.92 ($n=13$)
GA	19.46	100.51 ± 10.12 ($n=13$)	3.80 ($n=5$)	10.07 ($n=13$)
4OMGA	21.84	111.76 ± 8.02 ($n=13$)	1.38 ($n=4$)	7.17 ($n=13$)

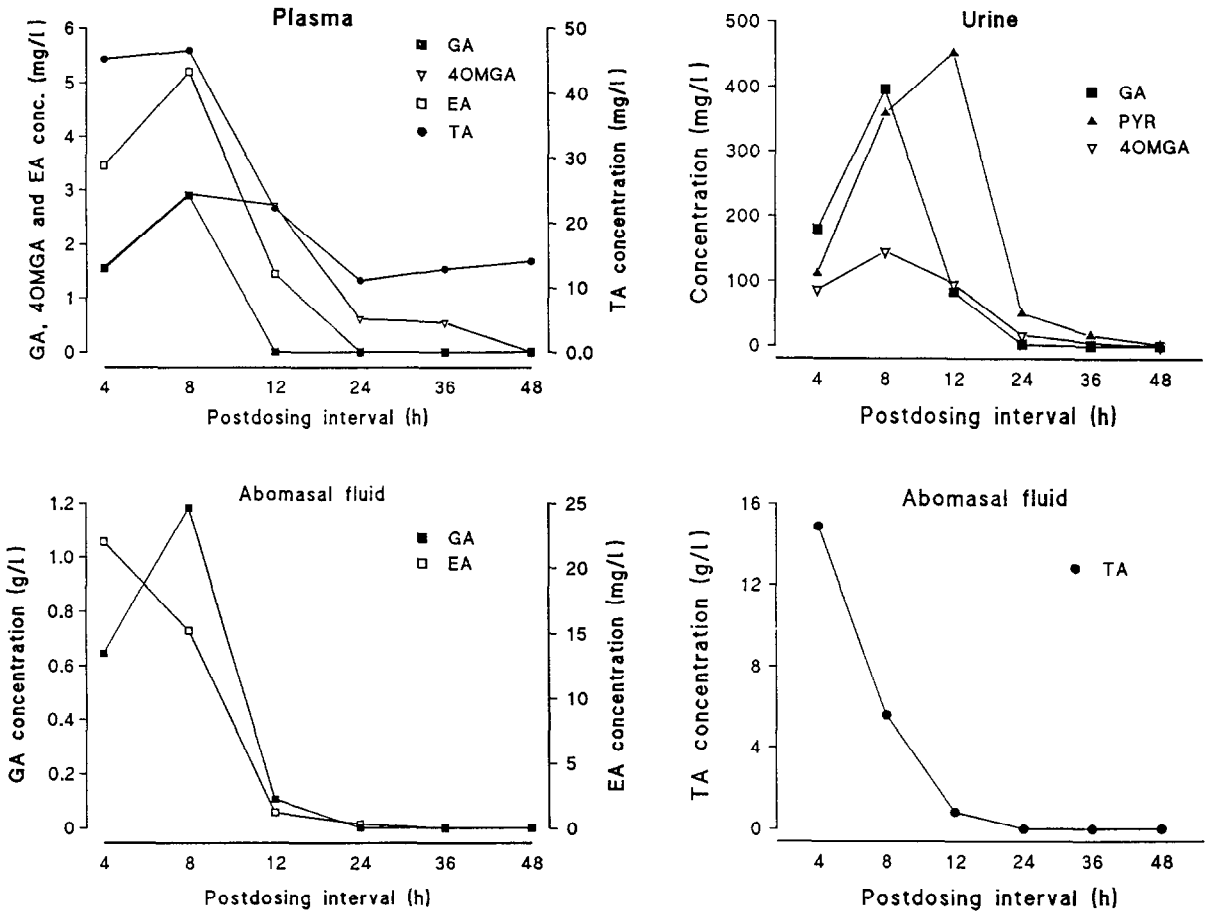

Fig. 6. TA, GA, PYR, 4OMGA and EA concentrations in sheep plasma, abomasal fluid and urine after abomasal dosing of TA at 1.0 g/kg body weight. Refer to Fig. 1 for abbreviations.

intestine as it passed down to the intestine from the stomach. It appeared that most of TA was absorbed and metabolised in 12 h following dosing of TA since TA concentrations in both plasma and abomasal fluid were dramatically reduced within 12 h and became undetectable in the abomasal fluid 24 h after dosing. Changes of EA content over time in plasma and abomasal fluid were similar to those of TA. In comparison to TA and GA concentrations in the abomasal fluid, the excretion of GA, PYR and 4OMGA in the urine was remarkably reduced after 24 h and was not detected 48 h after dosing of TA. PYR and 4OMGA have previously been found in both chicken urine and faeces after oral dosing of TA [21]. In our current studies, the observation that PYR was not found in plasma could be due to the rapid excretion of this compound through the kidney as large amounts of PYR were found in the urine. Booth *et al.* [8] reported the presence of GA, PYR and 4OMGA in rat urine after orally dosing with either TA or GA or intraperitoneal dosing of GA. Their results indicate that PYR and 4OMGA are the metabolic products of decarboxylation and methylation of GA by the liver. 2-O-Methylpyrogallol found in the rat urine by Booth *et al.* [8] was not determined in this study because the standard compound was not available.

In conclusion, the HPLC method described is a satisfactory procedure for the separation and measurement of TA and its phenolic metabolites in biological materials. It affords baseline separation of a mixture of ten phenolic compounds containing PYR, GA, CAT, RES, PA, 4OMGA, 4HBA, phenol, BA and EA. This method could be applied for the measurement of other hydrolysable tannins and phenolic compounds.

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