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Determination of gallic acid and its metabolites in human plasma and urine by high-performance liquid chromatography

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Abstract

Gallic acid occurs naturally in plants and has been found to be pharmacologically active as antioxidant, antimutagenic and anticarcinogenic agent. In this work, the metabolism of gallic acid in the human body was investigated. Two methods were developed for the identification and determination of gallic acid and its phenolic metabolites in human plasma and urine by reversed-phase high-performance liquid chromatography using UV detection and involving isocratic elution. One of these methods enables the simultaneous separation and determination of gallic acid (GA), 4-O-methylgallic acid (4OMGA), pyrogallol (PY), 2-O-methylpyrogallol (2OMPY) and resorcinol (RE) in biological fluids. This method is of interest because it allows the separation of a large number of phenolic compounds by isocratic elution using a solution of $4.4 \cdot 10^{-3}$ *M* phosphoric acid in water as mobile phase. The analysis time for this method, however, is not optimal (57 min). After oral administration of 50 mg GA, 4OMGA rapidly appeared in the plasma and urine besides unchanged GA. Other phenolic compounds, PY, 2OMPY and RE, were not detected. The second method was developed to determine GA and 4OMGA with a short analysis time (25 min). © 1998 Elsevier Science B.V.

Keywords: Gallic acid

1. Introduction

Gallic acid (GA) is widely distributed in plants. Bound forms of GA, notably epicatechin gallate or galloyl glucosides and tannic acids, are associated with and probably are the main source of free GA in foods [1].

For pharmaceutical purposes GA is employed as a reducing agent (Dermatol, Airol, bismuth salt of GA) [2] and as a homeopathic drug (Acidum gallicum) [1,3] and this substance has been recently recognised as possessing antiallergic, anti-inflammatory, antimutagenic and anticarcinogenic activities [1,4–8].

In spite of the health importance of GA, its metabolism and kinetics in the human body have not been investigated. In rats, rabbits and chickens the major urinary metabolite is 4OMGA and the secondary product is pyrogallol (PY) (conjugated and unconjugated) [1,9–13]. Small amounts of conjugated 2-O-methylpyrogallol (2OMPY) as a third metabolite of GA were also detected in rats [9,11]. In sheep resorcinol (RE) glucuronide is the major product of GA metabolism and the minor urinary metabolites are unconjugated PY and RE [14].

Various methods for determination of GA and other phenolic compounds in biological materials of animals have been reported using paper chromatography [10–12] and thin-layer chromatography [9,10],

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but these methods are nonspecific and nonsensitive. Gas chromatography (GC) offers high sensitivity for a wide range of phenolic compounds and has been used to determine GA and PY in bovine urine and serum samples [15]. However, it generally requires a derivatisation step before chromatography. Although high-performance liquid chromatography (HPLC) is the most commonly used method for the measurement of GA and other phenolic compounds in foodstuffs [16–18], there are few literature references concerning the determination of these compounds in the animal body [14,19] and no literature references about their determination in human blood, tissue or urine.

Zhu et al. [19] described an HPLC method for the determination of tannic acid and its phenolic metabolites (GA and some other phenolic compounds) in biological fluids collected from a sheep following abomasal dosing of tannic acid at 1.0 g/kg body mass. Our attempts to use that method for identification of GA and its phenolic metabolites in human plasma and urine after giving a single dose of 50 mg GA from two acidum gallicum tablets to a volunteer were unsuccessful.

In order to investigate if 4-O-methylgallic acid (4OMGA), PY, 2OMPY and RE (see Fig. 1 for structures), the well-known metabolites of GA in the animal body [1,9–14], could be formed in the human body too, a reversed-phase HPLC technique was developed which enables their separation and determination in human urine and plasma.

This method is of interest because it makes possible the separation of the above mentioned phenolic compounds by isocratic elution using a solution of $4.4 \cdot 10^{-3}$ *M* phosphoric acid in water as mobile phase. By this method it could be demonstrated that 4OMGA is the only phenolic metabolite of GA in the human body. However the time of the analysis is not optimal (57 min). Therefore another assay was developed which allows the separation and quantification of GA and 4OMGA within 25 min.

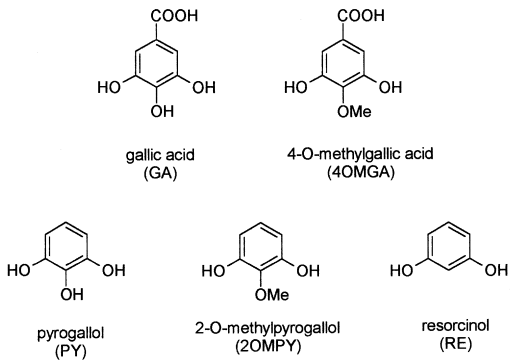

Fig. 1. Structures of the compounds used in this study.

2. Experimental

2.1. Reagents and standards

Phosphoric acid (Merck, Darmstadt, Germany) was used as pH modifier. Solvents were HPLC-grade (Merck). 4OMGA and 2OMPY were prepared according to published literature procedures [13,20,21] and their purity was established by spectroscopic methods. Other chemicals were purchased from Fluka (Neu-Ulm, Germany).

2.2. Equipment

A Merck and Hitachi high-precision pump Model L-6000 equipped with a 100-μl loop was used. The solutes were detected using a Gynkotek spectrophotometer SP-4 at 220 and 270 nm. Separation was carried out using a LiChrospher 100 RP-18 column (5 μm; 120 mm×4 mm I.D., Merck) with a guard column (RP-18; 4×4 mm, Merck). Chromatographic data were collected and recorded using a Merck and Hitachi D-2000 chromato-integrator. Degassing of HPLC mobile phases and some solutions (samples and HCl used for hydrolyses under argon) was carried out using a Bandelin Sonorex ultrasonic bath (MAGV, Rabenau-Londorf, Germany). Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.3. Collection of clinical samples

A healthy, female volunteer, 24-years-old, participated in this study which was reviewed and accepted by the ethics commission of the Justus Liebig University in Giessen. She followed a GA-free diet for 14 h, which consisted of white bread, cheese and water. Predose blood (collected into heparinized blood containers, KABE Labortechnik, Nuembrecht-

Elsenroth, Germany) and urine samples were collected. Blood was immediately centrifuged (at 1800 *g* for 10 min) to obtain plasma. Plasma and urine samples were stored at −18°C until analysed. A single dose of 50 mg GA (two acidum gallicum tablets) was administered orally with 200 ml of water. Plasma and urine samples were collected 90 min after administration and stored as described above. Collecting of urine continued for 24 h after administration for the determination of GA excretion.

2.4. Experimental procedures to determine GA, 4OMGA, PY, 2OMPY and RE (method 1)

2.4.1. Chromatographic separation (time of analysis: 57 min)

The mobile phase was $4.4 \cdot 10^{-3}$ *M* phosphoric acid in water. A flow-rate of 1.0 ml/min and room temperature were used throughout the study. Retention times and absorbance ratios (at two different wavelengths: 220 and 270 nm) against those of standards were used to identify the separated phenolic acids and to check their purity. Quantitative determinations were carried out by the external standard method.

2.4.2. Sample preparation

2.4.2.1. *Hydrolysis*. In order to eliminate the oxidation effect of air on PY and RE during acid hydrolysis (GA, 4OMGA and 2OMPY were stable during acid hydrolysis under air), the hydrolyses were carried out under air exclusion. 2 ml of plasma or urine samples were mixed with an equal volume of 1 *M* hydrochloric acid (the samples and HCl had been degassed in an ultrasonic bath) in round-bottom flasks and hydrolysed in a boiling water bath under argon for 30 min. After hydrolysis the samples were cooled to room temperature and extracted under argon.

2.4.2.2. *Extraction*. All samples, hydrolysed and nonhydrolysed, were subjected to extraction twice with a fourfold volume of ethyl acetate. The organic phase of processed urine samples was pipetted in round-bottom flasks and the combined ethyl acetate extracts evaporated to dryness under vacuum with a rotary evaporator, always keeping the bath temperature under 35°C. The plasma samples were centrifuged at 1800 *g* for 10 min to separate the organic fractions and the organic layers were dried in round-bottom flasks, as described for the urine samples.

Each extract was redissolved in HPLC mobile phase (0.5 ml for 2 ml plasma, 5 ml for 2 ml urine) before HPLC analysis.

2.4.3. Stock solutions

A mixture containing 500 mg/l of GA, 4OMGA, PY, 2OMPY and RE dissolved in $4.4 \cdot 10^{-3}$ *M* H_3PO_4 was used as the standard solution. Stock plasma and urine solutions were prepared in a range of 0.02–100.00 mg/l of standard solution. The solutions were stored in 1-ml vials at −18°C.

2.4.4. Standard curves

Duplicate standard curves were prepared by spiking predose plasma and urine with known amounts of standard solution prior to the preparation procedure (hydrolysis and extraction). Concentrations of GA, 4OMGA, PY, 2OMPY and RE were 0.05, 0.10, 0.50, 1.00, 5.00, 10.00, 50.00 and 100.00 mg/l.

2.4.5. Recovery

The recoveries of GA, 4OMGA, PY, 2OMPY and RE were determined using spiked predose plasma and predose urine at three different concentrations (Tables 2 and 3) of each substance. Recoveries were estimated by comparing the mean peak areas of hydrolysed and extracted spiked plasma and urine samples to the mean peak areas of equivalent aqueous standard solutions.

2.4.6. Accuracy and precision

Inter-day and intra-day variability studies were performed by spiking predose plasma or urine with three different concentrations of GA, 4OMGA, PY, 2OMPY and RE (Tables 2 and 3) prior to the sample preparation procedure (hydrolysis and extraction). Four samples at each concentration were injected on each of four consecutive days.

2.5. Experimental procedures to determine GA and 4OMGA (method 2)

2.5.1. Chromatographic separation (time of analysis: 25 min)

The chromatographic separation of GA and 4OMGA was achieved with a mobile phase consisting of water–acetonitrile (97.5:2.5, v/v) modified with 0.025% phosphoric acid. A flow-rate of 1.0 ml/min and room temperature were used. The identification and quantification of the compounds was performed as described in Section 2.4.1.

2.5.2. Sample preparation

Two ml of 1 *M* sulphuric acid was added to 2 ml of plasma or urine in glass tubes. The tubes were put in a boiling water bath and the samples were hydrolysed for 30 min [16] and then cooled in a cold water bath. These samples and nonhydrolysed samples were extracted as described in Section 2.4.2.2.

2.5.3. Stock solutions

A mixture containing 500 mg/l of GA and 500 mg/l 4OMGA dissolved in $4.4 \cdot 10^{-3}$ *M* H_3PO_4 was used as the standard solution. Stock plasma and urine solutions were prepared in a range of 0.02–100.00 mg/l of standard solution. The solutions were stored in 1-ml vials at −18°C.

2.5.4. Standard curves

Triplicate standard curves were prepared by spiking predose plasma and urine with known amounts of GA and 4OMGA prior to the sample preparation procedure. Concentrations of both GA and 4OMGA were 0.05, 0.1, 0.50, 1.00, 5.00, 10.00, 50.00 and 100.00 mg/l.

2.5.5. Recovery, accuracy and precision

The recovery as well as inter-day and intra-day variability studies for GA and 4OMGA were performed as described in Sections 2.4.5 and 2.4.6. The concentrations used are given in Tables 5 and 6.

3. Results and discussion

The well-known metabolic reactions of GA in animals are conjugation [1,14], 4-O-methylation [1,9–13], decarboxylation [9–13] and dehydroxylation [14].

In this work a preliminary study was carried out to identify GA and its possible metabolites, 4OMGA, PY, 2OMPY and RE, which could be formed through the reactions mentioned above in the human body. We developed a simple and accurate HPLC method (method 1) for the simultaneous separation of these compounds in human plasma and urine.

All five compounds in the standard mixture were completely separated within 55 min as shown in Fig. 2a. Standard curves were linear over the range 0.10–100.00 mg/l for PY and RE and 0.05– 100.00 mg/l for other substances (GA, 4OMGA and 2OMPY) in both plasma and urine, and the model $y=a+bx$ was therefore used to describe the calibration curves,

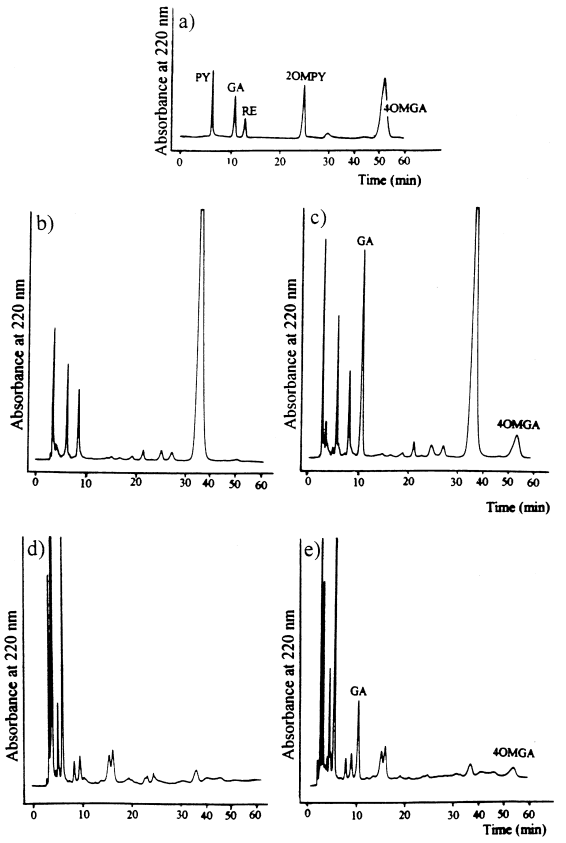

Fig. 2. Chromatograms obtained by HPLC method 1 of (a) standards and of (b, c, d, e) under argon hydrolysed samples. (b) Blank urine; (c) urine, 90 min after administration of GA; (d) blank plasma; (e) plasma, 90 min after administration of GA.

Table 1
Calibration curves for the five components determined in predose plasma and urine by method 1

Components	Calibration curves[a]			
	In plasma	r^2	In urine	r^2
PY	$y=-0.004+(2.23\cdot10^{-5})x$	0.997	$y=0.003+(2.10\cdot10^{-5})x$	0.998
GA	$y=-0.007+(1.41\cdot10^{-5})x$	0.998	$y=-0.006+(1.30\cdot10^{-5})x$	0.999
RE	$y=-0.003+(3.22\cdot10^{-5})x$	0.998	$y=-0.008+(3.11\cdot10^{-5})x$	0.997
2OMPY	$y=-0.010+(2.95\cdot10^{-5})x$	0.998	$y=-0.004+(2.84\cdot10^{-5})x$	0.999
4OMGA	$y=-0.009+(2.32\cdot10^{-5})x$	0.999	$y=-0.001+(2.17\cdot10^{-5})x$	0.999

[a] The calibration curves were determined at 220 nm.

where y is component concentration (mg/l) and x peak area. The typical calibration equations for each component are listed in Table 1.

The intra-day and inter-day variabilities are presented in Tables 2 and 3. In all cases the coefficient of variation (C.V.) was less than 15.22%. Recoveries for all substances exceeded 67.9%.

For PY and RE were the limit of detection (LOD) and the limit of quantification (LOQ) after hydrolysis and extraction of plasma or urine 0.05 and 0.1 mg/l, and for the other substances, GA, 4OMGA and 2OMPY, 0.025 and 0.05 mg/l, respectively.

The chromatograms obtained by this method of the under argon hydrolysed samples (the predose

Table 2
Intra-day variabilities and recoveries of PY, GA, RE, 2OMPY and 4OMGA in predose plasma and predose urine determined by method 1

Component added in plasma or urine (mg/l)	Mean values of four findings					
	Plasma			Urine		
	Observed (mg/l)	Recovery (%)	C.V. (%)	Observed (mg/l)	Recovery (%)	C.V. (%)
PY						
0.51	0.40	78.43	12.11	0.43	84.31	9.49
10.20	7.56	74.12	8.54	8.94	87.65	7.26
51.00	46.59	91.35	7.89	46.85	91.86	8.13
GA						
0.53	0.47	90.57	7.20	0.51	96.23	8.33
10.60	10.02	94.53	3.25	10.08	95.09	3.95
53.00	49.36	93.13	2.86	52.60	99.24	2.71
RE						
0.53	0.39	73.58	12.77	0.36	67.92	10.59
10.60	7.94	74.91	11.14	8.28	78.11	9.38
53.00	42.55	80.28	11.01	42.23	79.68	6.26
2OMPY						
0.51	0.48	94.12	6.14	0.50	98.04	6.17
10.20	9.54	93.53	2.59	10.09	98.92	3.63
51.00	47.93	93.98	3.21	49.35	96.76	3.28
4OMGA						
0.56	0.52	92.86	7.46	0.55	98.21	6.48
11.10	9.87	88.92	3.54	11.09	99.91	2.91
55.50	52.72	94.99	3.62	54.99	99.08	2.86

Table 3
Inter-day variabilities and recoveries of PY, GA, RE, 2OMPY and 4OMGA in predose plasma and predose urine determined by method 1

Component added in plasma or urine (mg/l)	Mean values of 16 findings					
	Plasma			Urine		
	Observed (mg/l)	Recovery (%)	C.V. (%)	Observed (mg/l)	Recovery (%)	C.V. (%)
PY						
0.51	0.38	74.51	15.21	0.42	82.35	11.76
10.20	8.32	81.57	14.38	9.00	88.23	11.24
51.00	45.08	88.39	10.56	46.23	90.65	13.20
GA						
0.53	0.47	92.45	8.81	0.52	98.11	10.81
10.60	9.90	93.40	3.94	10.55	99.53	4.11
53.00	50.21	94.74	3.85	52.12	98.34	3.04
RE						
0.53	0.36	67.92	14.73	0.38	71.70	12.44
10.60	8.39	79.15	14.87	8.07	76.13	10.76
53.00	43.21	81.53	12.66	43.43	81.94	12.95
2OMPY						
0.51	0.49	96.08	9.23	0.47	92.16	8.76
10.20	9.61	94.22	3.99	9.81	96.18	4.02
51.00	48.03	94.18	3.57	48.63	95.35	3.71
4OMGA						
0.56	0.51	91.07	8.88	0.56	100.00	9.84
11.10	10.34	93.15	3.80	10.91	98.29	4.35
55.50	52.53	94.65	3.72	55.01	99.12	3.26

plasma and urine samples and the samples collected 90 min after administration of GA) are shown in Fig. 2b–e.

The experimental results showed that GA given to the volunteer in 50 mg quantity, was found only as 4OMGA and unchanged GA in plasma and urine. No additional metabolite was evident either before or after the hydrolysis (Fig. 2). Samples containing GA and 4OMGA can be hydrolysed with 1 *M* H_2SO_4 under air because they are stable when treated under these conditions (Tables 5 and 6).

A second HPLC method (method 2) with a shorter analysis time (25 min) also was developed to determine GA and its metabolite 4OMGA.

The chromatograms of hydrolysed (under air) and nonhydrolysed plasma and urine samples obtained by method 2 are shown in Figs. 3 and 4.

We introduced this method (method 2) for quanti-

Table 4
Calibration curves for GA and 4OMGA determined in predose plasma and urine by method 2

Components	Calibration curves[a]			
	In plasma	r^2	In urine	r^2
GA	$y=-0.007+(1.34\cdot10^{-5})x$	0.999	$y=-0.003+(1.22\cdot10^{-5})x$	0.999
4OMGA	$y=-0.006+(2.27\cdot10^{-5})x$	0.999	$y=-0.005+(2.12\cdot10^{-5})x$	0.999

[a] The calibration curves were determined at 220 nm.

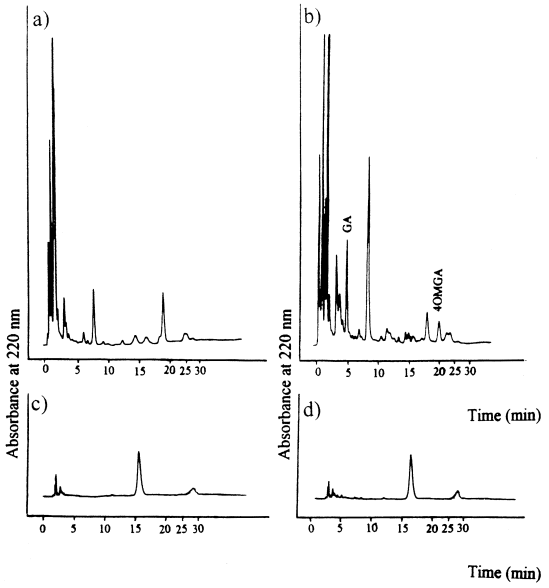

Fig. 3. Chromatograms obtained by HPLC method 2 of plasma samples. (a) Hydrolysed blank plasma; (b) hydrolysed plasma, 90 min after administration of GA; (c) nonhydrolysed blank plasma; (d) nonhydrolysed plasma, 90 min after administration of GA.

fication of GA and its metabolite, 4OMGA. The standard curves were linear over the range investigated (Table 4). The intra-day and inter-day variabilities are presented in Tables 5 and 6. In all cases

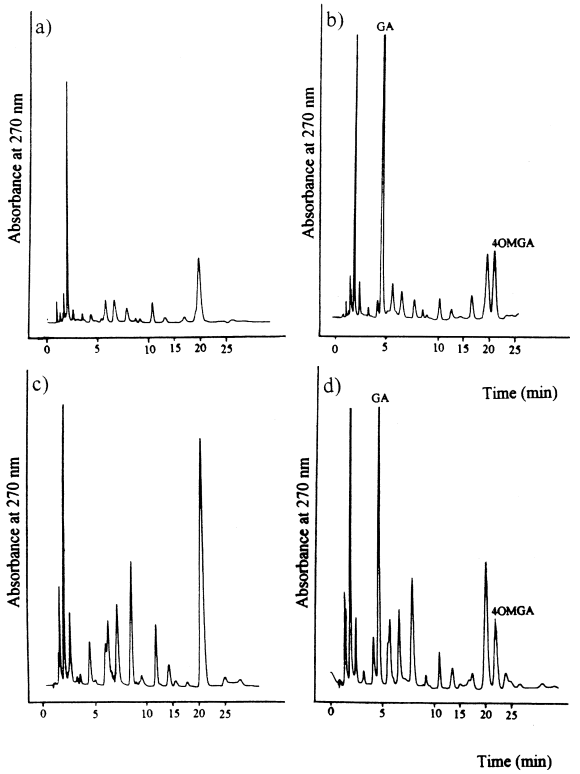

Fig. 4. Chromatograms obtained by HPLC method 2 of urine samples. (a) Hydrolysed blank urine; (b) hydrolysed urine, 90 min after administration of GA; (c) nonhydrolysed blank urine; (d) nonhydrolysed urine, 90 min after administration of GA.

Table 5
Intra-day variabilities and recoveries of GA and 4OMGA in predose plasma and predose urine determined by method 2

Component added in plasma or urine (mg/l)	Mean values of four findings					
	Plasma			Urine		
	Observed (mg/l)	Recovery (%)	C.V. (%)	Observed (mg/l)	Recovery (%)	C.V. (%)
GA						
0.53	0.49	92.45	7.36	0.51	96.23	8.47
10.60	9.84	92.83	3.21	10.52	99.24	3.29
53.00	48.11	90.77	3.08	52.36	98.79	2.71
4OMGA						
0.56	0.52	92.86	8.79	0.54	96.43	6.13
11.10	10.11	91.08	3.25	10.93	98.47	3.83
55.50	51.78	93.30	3.91	55.61	100.20	2.46

Table 6
Inter-day variabilities and recoveries of GA and 4OMGA in predose plasma and predose urine determined by method 2

Component added in plasma or urine (mg/l)	Mean values of 16 findings					
	Plasma			Urine		
	Observed (mg/l)	Recovery (%)	C.V. (%)	Observed (mg/l)	Recovery (%)	C.V. (%)
GA						
0.53	0.48	90.57	9.01	0.53	100.00	12.11
10.60	9.82	92.64	3.86	10.80	101.89	3.06
53.00	49.85	94.06	3.97	51.88	97.89	2.95
4OMGA						
0.56	0.51	91.07	10.17	0.55	98.21	10.26
11.10	10.00	90.09	4.76	11.11	100.09	4.99
55.50	52.96	95.42	5.07	54.22	97.69	3.51

the C.V.s were less than 12.5%. Recoveries for both GA and 4OMGA exceeded 90%. The LOD and LOQ of GA and 4OMGA in both human plasma and human urine were 0.025 and 0.05 mg/l, respectively.

Table 7
Concentrations of GA and 4OMGA in plasma and urine 90 min after oral administration of 50.08±1.54 mg GA

	Mean values of four findings	
	Plasma (mg/l)	Urine (mg/portion=mg/30 ml)
Before hydrolysis		
GA	nd[a]	1.301±0.039
4OMGA	nd	2.543±0.084
After hydrolysis		
GA	0.305±0.008	3.83±0.11
4OMGA	0.422±0.010	4.36±0.15

[a] nd=Not detected.

The concentrations of GA and 4OMGA in the urine and the plasma 90 min after the application are given in Table 7.

To determine the amounts of GA excreted (as unchanged GA or 4OMGA), the urine samples collected over a 24 h period were analysed using HPLC method 2 before and after hydrolysis with 1 *M* H_2SO_4 under air. The results are given in Table 8.

It was shown that after 24 h 37.14±1.80% of the GA administered was excreted. The last urine sample collected in this study did not contain detectable amounts of GA and 4OMGA.

The extract of nonhydrolysed plasma sample after application of GA contained no GA and no 4OMGA (Fig. 3). Possible explanations are the protein binding of these substances in blood [22,23] or their conjugation. Conjugation could also explain the greater concentration of GA and 4OMGA determined in hydrolysed urine compared to nonhydrolysed urine (Tables 7 and 8) [1,24].

Table 8
Amounts of GA excreted over 24 h after administration of 50.08±1.54 mg GA

	Mean values of four findings		
	Unchanged GA excreted (mg)	4OMGA excreted (mg)	Total[a] GA excreted (mg)
Before hydrolysis	1.96±0.15	7.85±0.39	9.21±0.39
After hydrolysis	5.98±0.31	13.66±0.68	18.60±0.70

[a] (Unchanged GA excreted)+0.924(4OMGA excreted).

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