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Determination of 2-hydroxyphenylacetic acid (2HPAA) in urine after oral and parenteral administration of coumarin by gas-liquid chromatography with flame-ionization detection

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Abstract

The urinary excretion of 2-hydroxyphenylacetic acid (2HPAA) was studied in human volunteers after oral and parenteral doses of coumarin. The presence of 2HPAA in the urine was confirmed by gas chromatography mass spectroscopy (GC MS). Mass spectra of reference material and samples are presented. The determination of 2HPAA was carried out by GC with flame-ionization detection. Prior to analysis samples were extracted into ethyl ether and the analytes were derivatized with trimethylphenylammonium hydroxide. A calibration range from 0.3 to 150 μg ml^{-1} was established using 3-hydroxyphenyl acetic acid (3HPAA) as an internal standard. On average less than 10% of the coumarin administered were excreted into the urine in the form of 2HPAA.

Keywords: 2-Hydroxyphenyl acetic acid; Coumarin; Urinary excretion; Gas chromatography

1. Introduction

Coumarin (5,6-benzo-α-pyrone) has been postulated to inhibit enzymes involved in carcinogenesis, e.g. DNA-specific poly(ADP ribose)-polymerase [1]. The possible use of coumarin in oral chemotherapy would require detailed knowledge as to its mechanism of action and its adverse effects. It is well known that coumarin undergoes extensive first-pass metabolism in various species including man [2–4] and exhibits hepatotoxic effects in some but not in all species [2]. The pharmacology of coumarin has recently been reviewed with emphasis on metabolism and analytical techniques [5]. Data from the literature show that the pharmacokinetics of coumarin was usually studied with doses in the 200 mg range. Moreover, there are contradictory reports as to the contribution to the overall excretion of the secondary metabolite 2-hydroxyphenylacetic acid (2HPAA) which was estimated to account for 1–6% of the dose by one group [4] and could not be detected by another [6].

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In our laboratory a study was conducted in human volunteers with the aim to investigate the pharmacokinetics of coumarin after high single oral doses of 1 and 2 g, respectively in comparison with a 250 mg intravenous injection. The concentrations of coumarin, 7-hydroxycoumarin, 7-hydroxycoumarin glucuronide and 3-hydroxycoumarin have been analysed in plasma and urine by standard techniques and the results have been reported elsewhere [7,8]. Additional efforts had to be made in order to determine the concentrations of 2HPAA in the urine samples from this study. We report here a gas chromatography (GC) procedure for the analysis of 2HPAA in urine together with data on 2HPAA excretion into the urine after oral and parenteral administration of coumarin.

2. Experimental

2.1. Materials

Coumarin was obtained from Schaper and Brümmer (Salzgitter, Germany). 2HPAA and 3HPAA as an internal standard were purchased from Sigma (Deisenhofen, Germany). Diethyl ether, hydrochloric acid, anhydrous sodium sulphate and methanol were received from Merck (Darmstadt, Germany). Trimethylphenylammonium hydroxide (TMAH) (0.25 mol l^{-1} in methanol) was from Macherey and Nagel (Düren, Germany). All reagents were of analytical grade. Twice distilled water was used in all experiments. Blank urine was collected on an as needed basis from volunteers who did not use any medication.

2.2. Chromatography

The chromatographic system consisted of an Hewlett-Packard Model 5890 gas chromatograph equipped with a Megabore DB/1 fused-silica capillary (20 m, 0.53 mm id, 1.5 μm film thickness) delivered by J and W/Fisons (Mainz, Germany). Splitless on-column injection was used. The injector was kept at a temperature of 270°C. The carrier gas was helium at a flow rate of 1 ml min^{-1}. The chromatographic separation was achieved with a temperature program for the column oven: initially the temperature was kept at 100°C until 5 min after injection and was then increased linearly to 310°C with a rate of 20°C min^{-1}. The final hold-time was 5 min. Typically 1 μl was injected into the GC system. A flame-ionization detector (FID) was used in most chromatographic runs. The detector output was recorded and reported with a HP 3396A integrator (Hewlett-Packard, Böblingen, Germany).

During method development the FID was replaced with a mass-selective detector (Hewlett-Packard Model 5970) connected to the GC system by a capillary direct interface. The mass spectrometer was calibrated with pentafluorotributylamine (PFTBA) at masses 69, 219 and 502. Ionization was carried out by electron impact at 70 eV. Spectra were obtained by scanning the mass range 30–180 a.m.u at a scan rate of 1 s^{-1}.

2.3. Urine samples

Urine samples from ten human volunteers who received three coumarin doses (2 g orally, 1 g orally and 250 mg intravenously) in a randomized cross-over study [7] were collected in four fractions in addition to a pre-dosing blank: 0–6, 6–12, 12–24 and 24–48 h after administration of coumarin. The volume of each fraction was recorded, a 10 ml aliquot of each fraction was adjusted to pH 4.5 with 0.1 N hydrochloric acid and kept frozen at −20°C until further analysis.

2.4. Sample preparation

After thawing samples were centrifuged ($2700 \times g$, 10 min) to remove any particles. Then 100 μl 1 N hydrochloric acid and 50 μl of an aqueous solution containing 300 μg ml^{-1} 3HPAA were added to 1 ml of urine in a polypropylene tube. The mixture was consequently extracted into 5 ml of ethyl ether on a reciprocating shaker for 10 min. After phase separation the ether phase was carefully removed and dried over 500 mg sodium sulfate. The solution was pipetted into a new tube and the solvent evaporated under a gentle stream of nitrogen. The residue was redissolved in 300 μl of methanol and then transferred

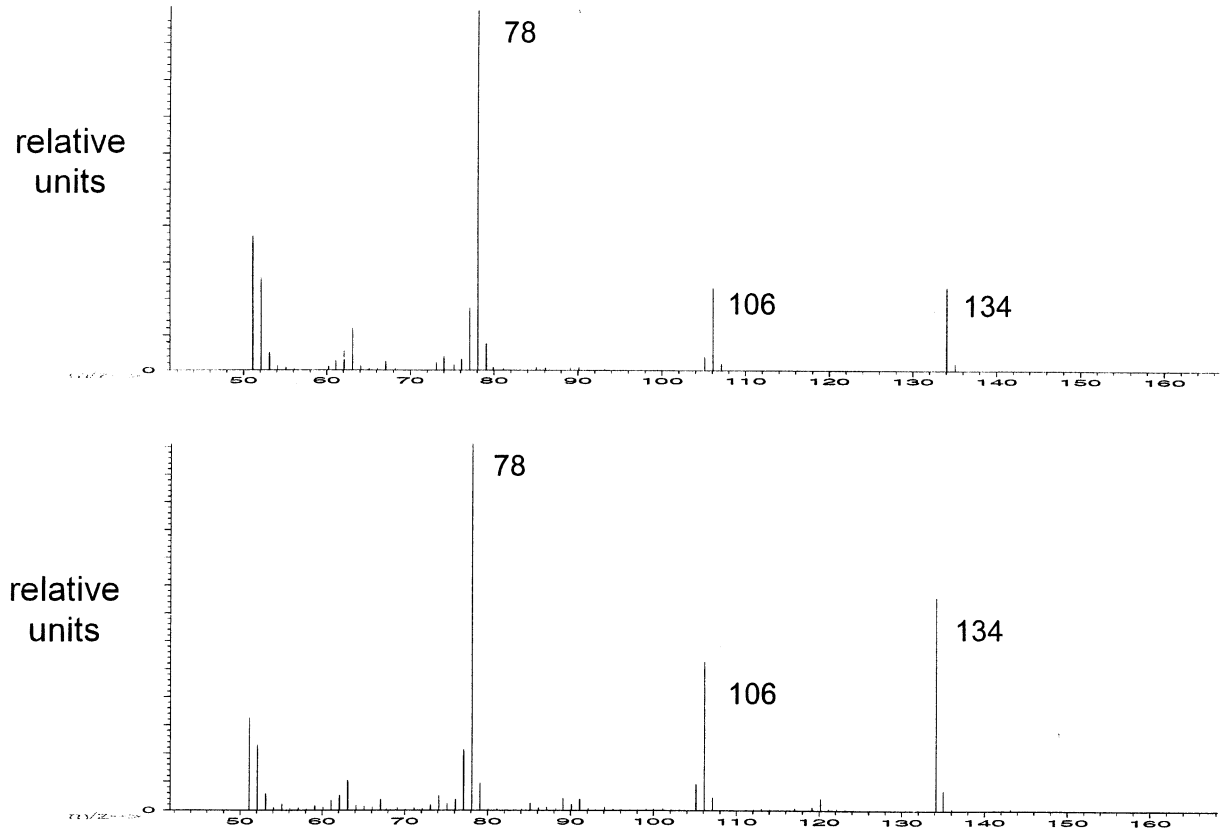

Fig. 1. Mass spectra of 2HPAA. Upper panel: reference dissolved in methanol; lower panel: urine sample 4–8 h after 1 g oral coumarin; the sample work-up was without derivatization step.

into a tapered glass reaction vessel equipped with a teflon lined gas proof screw cap. The methanol was evaporated again under nitrogen. Immediately prior to GC analysis 60 μl TMAH solution was added per sample, the screw cap fastened and the samples kept at 100°C for 15 min in a heating block. After derivatization the samples were allowed to cool down for another 10 min and were then chromatographed.

2.5. Calibration and calibration samples

Working standard solutions were prepared containing 3000, 300 and 30 μg ml^{-1} 2HPAA in water. Appropriate aliquots were added to blank urine to result in 1 ml samples with 0.3, 1.5, 3.0, 15.0, 30.0 and 150 μg ml^{-1} 2HPAA. In addition a blank urine without analytes was included in each series. The calibration samples were processed as described above. From the peak-height ratios (2HPAA/internal standard) and the corresponding concentrations a calibration function was obtained by least-squares linear regression. The squared concentrations were applied as weights [9].

3. Results

3.1. Qualitative analysis

A reference mass spectrum of 2HPAA and a spectrum from a chromatogram of a urine frac-

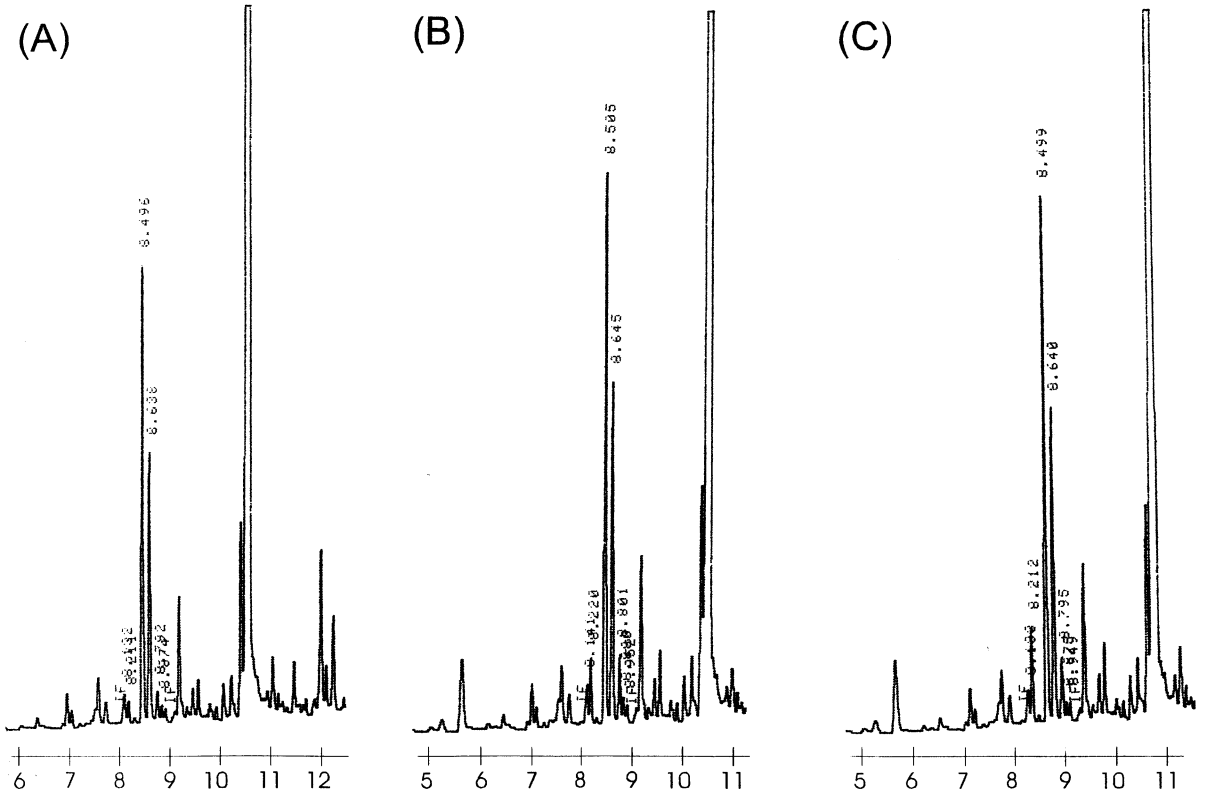

Fig. 2. Gas chromatograms with flame-ionization detection. (A) Gas chromatogram of urine blank; peak: 8.5 min, internal standard. (B) Gas chromatogram of 1.5 μg ml^{-1} calibration sample; peaks: 8.2 min, 2HPAA; 8.5 min internal standard. (C) Gas chromatogram of 3.0 μg ml^{-1} calibration sample; peaks: 8.2 min, 2HPAA, 8.5 min internal standard. All samples were processed as described in the text including derivatization (sensitivity: 64 mV full scale).

tion collected after administration of 1 g coumarin are shown in Fig. 1. The urine sample was processed as described above but without the final derivatization step. The spectra match well and provide retention time independent proof for the presence of 2HPAA. The mass spectra in Fig. 1 obviously lack the signal expected for the molecular ion (m/z 152) of 2HPAA. The compound is apparently dehydrated under intramolecular ester formation (m/z 134) at the elevated temperature in the injector block. The internal standard cannot undergo this particular reaction and could not be chromatographed satisfactorily without derivatization. Two further prominent fragment signals (m/z 106 and 78) are produced by successive loss of carbon monoxide.

3.2. Quantitative analysis

In Fig. 2 two chromatograms of a calibration sample and a blank are depicted. The sample work-up was as described including derivatization. The chromatograms show that the sample preparation procedure results in sufficiently clean samples to allow the quantitation of the peaks of interest. 2HPAA and 3HPAA, the internal standard, both have narrow symmetrical peaks and are well resolved. The retention index (Kovacs index) of 2HPAA was calculated to be 1220.

The calibration function $y = a*x + b$ (where y is the peak-height ratio and x the concentration) was linear over the range tested (0.3–150 μg ml^{-1}) with a slope $a = 0.043$ and an intercept $b = 0.060$ on the basis of 52 calibration samples.

A correlation coefficient of 0.9909 was calculated. The interday coefficients of variation were 3.3% at the 0.3 μg ml^{-1} level ($n = 4$), 3.0% at the 1.5 μg ml^{-1} level ($n = 12$), 3.4% at the 3.0 μg ml^{-1} level ($n = 9$), 4.6% at the 15 μg ml^{-1} level ($n = 9$), 3.2% at the 30 μg ml^{-1} level ($n = 11$) and 4.8% at the 150 μg ml^{-1} level ($n = 7$). Both compounds, 2HPAA and 3HPAA, were extracted from urine into ethyl ether with high efficiency. Calculated recoveries were above 90% in all instances.

The concentrations measured in the urine fractions of the volunteers after administration of coumarin were transformed into amounts. The total amounts of 2HPAA excreted over the study period of 48 h are reported in Table 1 as the percentage of the respective dose. 2HPAA could be measured in the urine of all subjects at all dose levels. However, the results show wide intersubject and intrasubject variability. With one exception the percentage of the dose excreted into the urine in the form of 2HPAA is well below 10%. After intravenous injection less 2HPAA is formed than after oral administration.

Table 1
Urinary excretion of 2HPAA in percentage of the dose administered

Subject	Dose administered		
	2 g oral	1 g oral	250 mg iv
1	5.55	1.79	3.62
2	1.55	2.57	2.29
3	5.90	4.14	0.31
4	1.14	2.99	1.12
5	4.92	2.11	0.34
6	3.00	4.55	1.06
7	8.68	2.86	1.34
8	13.54	5.08	3.45
9	4.32	7.03	1.05
10	2.73	2.05	0.57
Mean	5.13	3.52	1.52
SD	3.71	1.67	1.21
Median	4.62	2.93	1.09
Highest	13.54	7.03	3.62
Lowest	1.14	1.79	0.31

4. Discussion

Even though several reports have been published so far describing analytical methods for the determination of 2HPAA [4,6] we present here for the first time an absolute and retention time independent identification of this compound in human urine after administration of coumarin. The mass spectrum obtained after GC MS without derivatization is conclusive especially because of the specific intramolecular ester formation. Apart from this feature the spectrum shows a simple fragmentation pattern as one would expect for a small aromatic molecule.

The cumulative urinary excretion of 2HPAA over 48 h found in this study confirms the result of Shilling et al. [4] that 2HPAA is a minor metabolite of coumarin, regardless of dose. The increased formation of 2HPAA after oral administration might be explained by the presence of a first-pass effect. Treatment of a genuine urine sample with 6 N hydrochloric acid at 100°C for 1 h did not produce an increased GC signal for 2HPAA. Therefore, there is no indication as to the presence of 2HPAA conjugates.

Initially attempts were made to analyse for 2HPAA by HPLC with UV detection. However, this analytical technique appeared to be more prone to interference from matrix constituents. Therefore complex sample preparation protocols were required which were accompanied by inevitable losses and consequently affected method sensitivity. In contrast, the GC method reported here is simple and straightforward with an efficient sample preparation step. It appears that gas chromatography after suitable derivatization can be applied advantageously in the analysis of small aromatic carboxylic acids. The derivatization step was introduced in order to synthesize derivatives of the analyte and the internal standard with better gas chromatographic properties than the parent compounds. Again the reaction is simple and does not require additional catalysts, stoppers or post-derivatization clean-up as other alternative derivatization reactions do.

The limit of detection of the method was estimated to be 0.15 μg ml^{-1}. The calibration range of 0.3–150 μg ml^{-1} was found to be adequate, so that no sample dilution protocols had to be used.

No information is available from existing databases as to the pharmacological and toxic properties of 2HPAA itself. Only recently it was reported that 2HPAA inhibits aldose reductase activity [10].

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