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Studies on hypoxoside and rooperol analogues from *Hypoxis rooperi* and *Hypoxis latifolia* and their biotransformation in man by using high-performance liquid chromatography with in-line sorption enrichment and diode-array detection

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Abstract

Methanol extracts of the corms of *Hypoxis rooperi* and *H. latifolia* were studied for their hypoxoside content by an in-line sorption enrichment HPLC technique [Kruger et al., J. Chromatogr., 612 (1993) 191]. Hypoxoside is the trivial name for (E)-1,5-bis(3′-hydroxy-4′-O-β-D-glucopyranosyl-phenyl) pent-1-en-4-yne and rooperol the aglucone obtained from β-glucosidase treatment. Hypoxoside and rooperol analogues containing 4, 3 and 2 hydroxyl groups resolved as separate peaks with the proportion of the latter two markedly higher in *H. latifolia* than in *H. rooperi*. After oral ingestion of hypoxoside by humans, no hypoxoside or rooperol appeared in the serum. Only rooperol was present in the faeces. The serum and urine contained at least three phase II metabolite peaks. Selective enzyme hydrolysis showed that they represent the diglucuronide, disulfate and glucuronide–sulfate conjugates of all three rooperol analogues.

1. Introduction

The plant family *Hypoxidaceae* includes several genera, of which the genus *Hypoxis* has been of medicinal interest [1]. Extracts of its corms have been ingested by man for a diversity of ailments including cancer [2].

Hypoxoside is the trivial name for (E)-1,5-bis(3′-hydroxy-4′-O-β-D-glucopyranosylphenyl) pent-1-en-4-yne which is the major norlignan diglucoside isolated from *Hypoxis obtusa* Bush [3]. Hypoxoside has also been isolated from *Hypoxis rooperi* T. Moore and partial synthesis of its aglucone, rooperol and analogues of rooperol, has been undertaken [4–6].

A high-performance liquid chromatographic (HPLC) technique was recently developed for the quantitative and qualitative determination of a broad range of xenobiotics in biological media [7]. The method involves a novel approach to the direct introduction of the sample (serum, urine and other aqueous media) onto an in-line extraction pre-column. Analytes are selectively and quantitatively concentrated before column

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switching. The method is particularly useful for toxicological screening [7] and pharmacokinetic studies in situations where patients receive several medicines [8]. The method has also been found useful in our studies on the anticancer potential of hypoxoside. The aim of this paper is to describe the chromatographic behaviour of hypoxoside and rooperol analogues containing 2, 3 and 4 hydroxyl groups and to demonstrate application of the method in the chemical characterization of phase II biotransformation products found in the serum and urine of man.

2. Experimental

2.1. Chemicals

HPLC grade water was prepared from distilled deionised water by filtration through a Millipore system (Bedford, MA, USA). HPLC solvents were from Burdick and Jackson (Muskegon, MI, USA). All other organic solvents and salts were pro analysi grade from Merck (Darmstadt, Germany). Arylsulfatase, β-glucuronidase and β-glucosidase were from Sigma (St. Louis, MO, USA) and ultrapure guanidine hydrochloride (guanidinium) from Bethesda Research Chemicals (Bethesda, MD, USA). Corms of *Hypoxis rooperi*[1] T. Moore and *Hypoxis latifolia*[1] Baker were provided by Essential Sterolin Products (Halfway House, South Africa).

2.2. Semi-preparative and quantitative analyses

The HPLC technique as described by Kruger et al. [7] was applied to the analyses of all samples. The in-line extraction pre-column was loaded with samples dissolved in 50 μl of ethanol or in up to 200 μl aqueous phase and made up to 500 μl with a solution containing 8.05 *M* guanidinium and 1.02 *M* ammonium sulfate.

2.3. Preparation of hypoxoside and analogues

Shredded *Hypoxis* corms were dehydrated in a convection oven at 70°C for 3.5 h and milled to a powder of 200 mesh; 12.5 kg of rhizome yielded 5 kg powder, which was extracted with 25 l of methanol at room temperature for 30 min. The filtered extract was evaporated under vacuum and yielded 1.5 kg of light brown powder. Further purification was by preparative HPLC. A Shimadzu (Kyoto, Japan) apparatus consisting of the following components was used: a SCL-8A system controler, two LC-8A mobile-phase delivery pumps, a SPD-6AV UV-Vis spectrophotometric detector, a FCV-130 AL reservoir inlet controler and a FCV-100B fraction collector.

The column (300 × 50 mm I.D.) was packed with C_8-bonded silica (Partisil Bioprep 20-μm C_8 75A) obtained from Whatman (Fairfield, NJ, USA). Isocratic delivery of mobile phase (acetonitrile–water, 15:85, v/v) was at a flow-rate of 100 ml/min with detection at 260 nm and collection of 250-ml fractions. Methanolic extract (5 g) dissolved in water (50 ml) was pumped directly on-column. Fractions containing mono-components were selected after analytical HPLC analysis, diluted with 3 parts of water and concentrated on C_{18}-bonded silica (40 μm, preparative grade). Elution from the sorbent material with methanol and subsequent evaporation of the eluates yielded up to 0.5 g of pure hypoxoside and its analogues.

2.4. Preparation of rooperol and analogues

The compounds were isolated after enzymatic hydrolysis of their respective purified glucosides obtained from *Hypoxis latifolia*. Each hypoxoside analogue (100 mg) was dissolved in 10 ml of 0.1 *M* acetate buffer (pH 5.5) to which 10 mg β-glucosidase was added and incubated for 4 h at 37°C. The hydrolysates were extracted with diethyl ether (1 × 5 ml), washed with water (1 × 5 ml), dried with anhydrous sodium sulfate and

[1] Hilliard and Burtt [9] consider *H. rooperi* and *H. latifolia* as synonyms for *H. hemerocallidea* Fischer and Meyer and *H. colchicifolia* Baker, respectively. We, however, prefer to use the synonyms since they appear in all the references consulted.

evaporated under nitrogen to yield 5–10 mg of product.

2.5. Preparative isolation of hypoxoside biotransformation products formed in man

Since hypoxoside is marketed as a herbal tea in South Africa it was not necessary to obtain ethical approval for its ingestion by healthy volunteers. However, ethical approval was obtained for a phase I clinical trial in cancer patients. Urine (5 l) collected from adults on oral hypoxoside (1–2 g/day) was passed through C_{18}-bonded silica (200 g, 40 μm) which was pre-treated with methanol and water followed by elution with water (400 ml), 10% methanol (v/v, 400 ml), 30% methanol (v/v, 800 ml) and finaly pure methanol (400 ml) and water (400 ml). The 30% methanol fraction was diluted with water (1600 ml) and again passed through the sorbent bed and eluted with water (400 ml) and finally with methanol (400 ml). The final methanol solution was evaporated under vacuum at 50°C to an aqueous residue which was lyophilised to yield 1 g of a mixture of hypoxoside metabolites and endogenous urinary components. A suitable volume of filtered aqueous solution of this residue (10 mg/ml) was injected onto the in-line pre-column analytical HPLC system such that fractions A, B and C (*vide infra*, Fig. 5) were collected at the column outlet. These fractions were rendered free of mobile phase buffer salts by standard reversed-phase sorption and lyophilised.

2.6. Enzymatic hydrolysis of hypoxoside urine metabolites

Purified urine metabolite fractions A, B and C were individually hydrolysed by incubation with β-D-glucuronidase and arylsulfatase. D-Saccharic acid 1,4-lactone (0.005 m*M*) and sodium sulfate (0.01 *M*) were used to inhibit any β-glucuronidase or arylsulfatase cross-activity in the respective enzyme preparations. Hydrolysis was in 0.1 *M* acetate buffer (pH 5.5) at enzyme and substrate concentrations of 1 mg/ml each incubated for 2 h at 37°C. The hydrolysates were extracted with diethyl ether which was evaporated to dryness under a stream of nitrogen.

2.7. Synthesis of rooperol disulfates

Pyridinium sulfate was prepared freshly as follows: dry pyridine (50 ml, 0.62 mol) was added slowly with stirring to a mixture of dry ethyl acetate (400 ml) and concentrated sulphuric acid (25 ml, 0.47 mol). The mixture was cooled and the precipitate of pyridinium sulfate, obtained by decantation, was rinsed with dry ethyl acetate and vacuum dried at 60°C to yield 85 g (100% yield) which was dissolved in dry dimethylformamide (250 ml) to provide a 1.92 *M* pyridinium sulfate reagent solution.

Rooperol and analogues (100 mg, 0.00355 mol) were dissolved in dry dimethylformamide (2.2 ml) to which pyridinium sulfate reagent (1.5 ml, 0.00288 mol) and acetic anhydride (0.3 ml, 0.00318 mol) were added and incubated at 90–100°C for 30 min, cooled and then poured into cold water (40 ml) while stirring. The solution was then passed through a C_{18}-bonded silica sorbent bed (4 g, 40 μm particle size) which was pre-treated with methanol and water followed by washing with 40 ml of water, 40 ml of 0.1 *M* sodium bicarbonate, 40 ml of water and 10 ml of methanol. The methanol eluate was evaporated under vacuum at 50°C to an aqueous residue which was lyophilised to yield 0.07 g of rooperol disulfate.

3. Results

3.1. Hypoxoside and rooperol analogues in H. rooperi and H. latifolia methanol extracts

Fig. 1 (upper part) shows a chromatogram of the methanol extract of *H. rooperi*. Hypoxoside (peak 1) is the major glucoside (confirmed by 2D NMR of a chromatographically homogeneous preparation). The lower part of Fig. 1 shows a chromatogram of a β-glucosidase treated sample. Peak 4 represents rooperol. During the course of hydrolysis an intermediate peak at

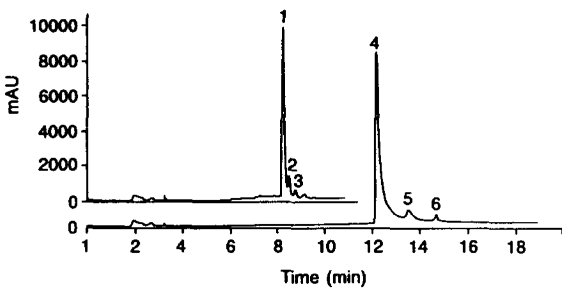

Fig. 1. Chromatograms of the hypoxoside (upper) and rooperol analogues (lower) present in *Hypoxis rooperi* methanol extract.

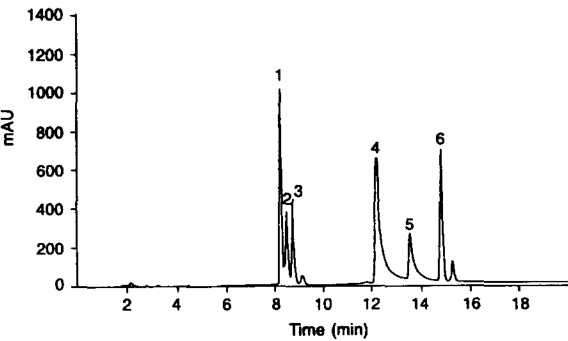

Fig. 2. Chromatograms of a 1:1 mixture of hypoxoside analogues found in the methanol extract of *Hypoxis latifolia* and their respective rooperol analogues obtained by β-glucosidase treatment. The two unnumbered peaks are new norlignans of which the structures will be reported elsewhere.

t_R = 10.1 min was observed which represented the monoglucoside obtuside A or B previously shown to be present in *H. obtusa* [10].

Fig. 2 shows a chromatogram of a 1:1 mixture of glucosides and their aglucones obtained from a methanol extract of *H. latifolia*. Since peak 3 was present in a much higher concentration than in *H. rooperi* it was purified to chromatographic homogeneity and its structure confirmed as the analogue of hypoxoside containing only two hydroxyl groups (bis-dehydroxyhypoxoside). Deglucosidation resulted in the formation of peak 6 (bis-dehydroxyrooperol). The coincidence of retention time and UV spectrum with 1,5-bis-(4'-hydroxyphenyl) pent-4-en-1-yne synthesized by Drewes et al. [5] confirmed its identity. Dehydroxyrooperol (peak 5) with a retention time intermediate between rooperol and bis-dehydroxyrooperol was inferred as having 3 hydroxyl groups on the two benzene rings. It was shown by Drewes et al. [4] to have the catechol group on the benzene closest to the alkene side of the hydrocarbon chain. Table 1 summarizes the structural features of the different analogues of hypoxoside and their respective aglucones present in the methanol extracts of *H. rooperi* and *H. latifolia*. A comparison of Figs. 1 and 2 shows that the dehydroxy and bis-dehydroxy analogues are present in larger concentrations in *H.*

Table 1
Phenolic pent-4-en-1-yne glucosides isolated from *Hypoxis* sp. and their respective aglucones

R1	R2	R3	R4	Trivial name	Peak No. in Figs. 1 and 2	Retention time (min)
O-β-D-glucose	OH	OH	O-β-D-glucose	Hypoxoside	1	8.2
O-β-D-glucose	H	OH	O-β-D-glucose	Dehydroxyhypoxoside	2	8.5
O-β-D-glucose	H	H	O-β-D-glucose	Bis-dehydroxyhypoxoside	3	8.8
OH	OH	OH	OH	Rooperol	4	12.2
OH	H	OH	OH	Dehydroxyrooperol	5	13.5
OH	H	H	OH	Bis-dehydroxyrooperol	6	14.6

latifolia than in *H. rooperi*. The unnumbered peaks in Fig. 2 represent a new norlignan of which the structure will be described elsewhere.

The UV spectra obtained from diode-array detection of the various hypoxoside and rooperol analogues are shown in Fig. 3. There is almost no difference in the spectra of the different glucosides while the dehydroxy and bis-dehydroxy aglucones display a relatively increased absorbance at 260 nm together with a decrease at 300 nm. However, general similarity in the spectral profiles of these substances indicate that they have chromophores with a common structure.

3.2. Hypoxoside biotransformation products in man

Fig. 4 shows chromatograms of the serum of a patient before and after ingestion of a methanol extract of *H. rooperi*. Three new peaks A, B and

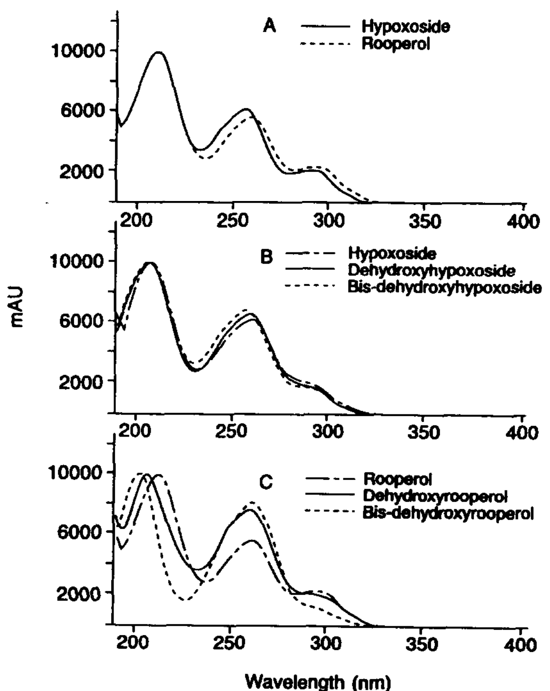

Fig. 3. Comparative UV absorption spectra from the diode-array detection of the hypoxoside analogues and their respective rooperol analogues (see Table 1 and Figs. 1 and 2).

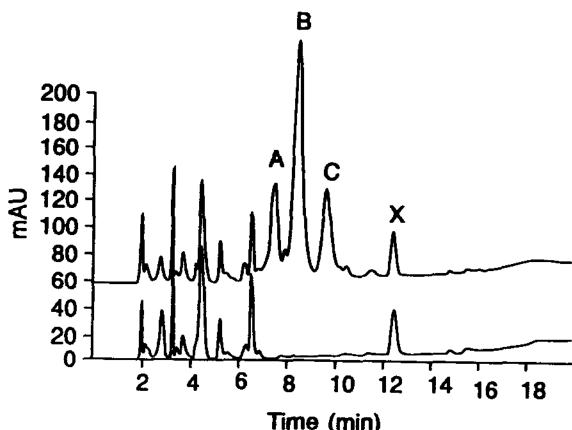

Fig. 4. The presence of biotransformation products A, B and C in the serum of an adult male (69 years) 2 h after oral ingestion of 1 g of hypoxoside. The retention times of the biotransformation products differ from the ingested hypoxoside (compare Tables 1 and 2). The lower chromatogram represents the serum before hypoxoside ingestion. The peaks eluting earlier than fraction A represent tryptophan, indole-related substances and caffeine while peak X is an unknown substance of dietary origin (see Kruger et al., [7]).

C are present in the post-hypoxoside serum with retention times of 7.6, 8.4 and 9.6 min which are distinctly different to the retention time of 8.2 min of the ingested hypoxoside. Fig. 5 (upper part) shows a parallel situation for the post-hypoxoside urine chromatogram. The shaded peaks A, B and C represent the biotransformation products of hypoxoside while the earlier eluting peaks are also present in pre-hypoxoside urine. Comparison of the UV diode-array detector spectra of the respective serum and urine peaks A, B and C (Fig. 6) show correspondence, confirming the presence of the same chromophore as for hypoxoside (Fig. 3). The lower part of Fig. 5 shows that the ingested hypoxoside had been converted quantitatively to rooperol (t_R = 12.2 min) in the subjects faeces. Rooperol is thus recognised as the chemical species available for absorption, and hence the precursor of the metabolites present in serum and urine. In order to establish the chemical nature of the rooperol metabolites urine fractions A, B and C were separately subjected to selective enzyme hydrolysis with arylsulfatase and/or β-glucuronidase.

The chromatogram of each purified urine

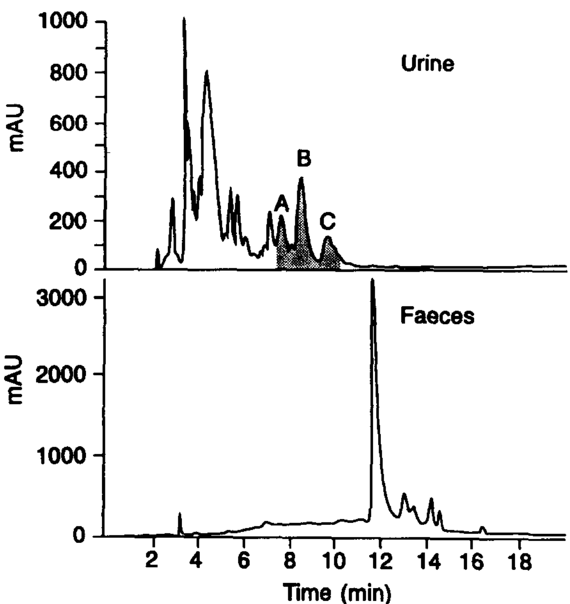

Fig. 5. Upper chromatogram: the presence of biotransformation products A, B and C in a 24-h urine sample of an adult male (69 years) after oral ingestion of 1 g of hypoxoside. The urine was concentrated on C_8-bonded silica as described in Experimental. The peaks eluting earlier than fraction A are endogenous substances with diode-array UV spectra differing from that of fractions A, B and C. Lower chromatogram: methanol extract of the faeces collected from the same subject 6 h after ingestion of 1 g of hypoxoside demonstrating that only rooperol (t_R = 12.2 min) is present.

fraction, A, B and C, is superimposed on the chromatogram of the respective products of enzyme hydrolysis in Fig. 7. It is clear that in each case variable ratios of rooperol, dehydroxyrooperol and bis-dehydroxyrooperol are present as the end-products of hydrolysis. The UV

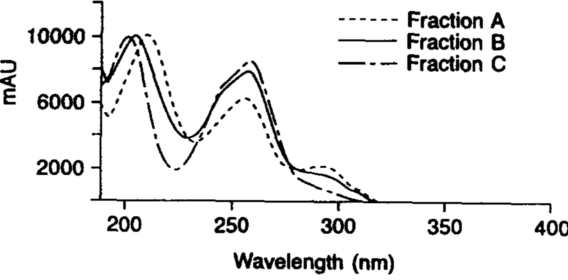

Fig. 6. Comparative UV diode-array detector spectra of purified biotransformation products A, B and C demonstrating the spectral features of the hypoxoside chromophore (compare with Fig. 3).

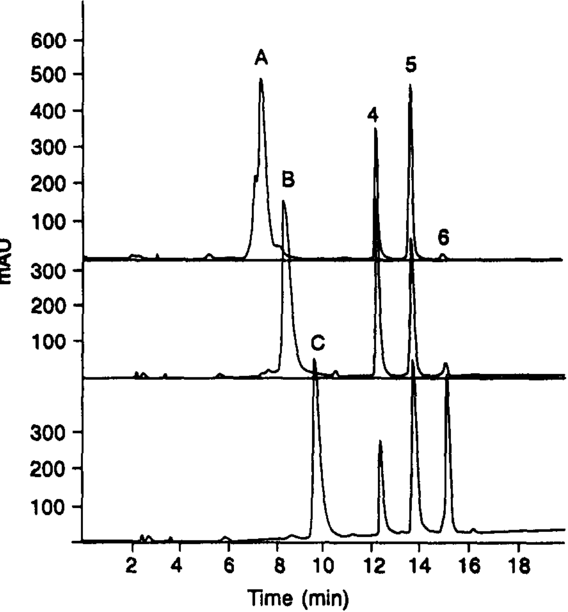

Fig. 7. A series of chromatograms demonstrating the selective enzyme hydrolysis of biotransformation products A, B and C purified from urine (see Fig. 5). In each case the chromatogram of the purified fractions A, B and C is superimposed on the chromatogram of the enzyme hydrolysis products. The t_R values and UV absorption spectra of the latter coincide with that of rooperol (4), dehydroxyrooperol (5) and bis-dehydroxy-rooperol (6) (see Table 1). Complete hydrolysis of fractions A and C was obtained with β-glucuronidase and arylsulfatase respectively, while fraction B required both enzymes for complete hydrolysis.

spectra of these peaks coincided with those shown in Fig. 3C and their retention times agreed with the authentic compounds found in the plant extracts (Figs. 1 and 2 and Table 1). In the case of fractions A and C full conversion was achieved by incubation with β-glucuronidase and arylsulfatase respectively which implies that fraction A is the di-β-glucuronide and fraction C the disulfate of all three rooperol analogues. When semisynthetic sulfates of rooperol and bis-dehydroxyrooperol were chromatographed together with fraction C, coincidence in retention time and spectra were found which supports the conclusion reached from the hydrolysis experiments.

With fraction B only partial deconjugation with either of the enzymes was found. β-Glucuronidase produced an intermediate peak

Table 2
Representative structures of the major biotransformation products of hypoxide in man after oral ingestion

Biological name	Retention time (min)
Rooperol di-glucuronate	7.6
Rooperol mono-sulfate mono-glucuronate	8.4
Rooperol di-sulfate	9.6

with $t_R = 11.0$ min (the residual monosulfate) and arylsulfatase produced a peak at $t_R = 9.6$ min (the residual monoglucuronide). When both enzymes were incubated with fraction B the conversion to rooperol analogues without intermediates, as shown in Fig. 7, was obtained. Table 2 summarizes the representative structures of the metabolites.

4. Discussion

Although it is known that the number of hydroxyl groups of hypoxoside can vary between 2 and 4 [5,6], it has never been demonstrated in a quantitative analytical system for the respective glucosides and aglucones from different *Hypoxis* species. We further feel that the analogues with different hydroxyl groups need to be distinguished by trivial names and therefore propose the prefixes dehydroxy- and bis-dehydroxy for the different analogues containing 3 and 2 hydroxyls respectively.

Figs. 1 and 2 show that the order of elution of the hypoxoside analogues remains the same as for their respective rooperol analogues, implying that they differ from each other by substituents on the aglucone, namely the hydroxyl groups. In terms of reverse phase chromatography retention mechanisms a decrease in hydroxyl groups results in increased retention times. This mechanism is amplified when the glucose moieties are removed. With the standardized HPLC system used we found a maximum variation of ± 0.3 min in retention times from day to day and when columns were changed. The relative retention times, however, remain constant for the different glucoside and aglucone analogues.

The HPLC method used by Betto et al. [11] to screen different genera and species of *Hypoxidaceae* for their contents of different norlignans showed that 11 different norlignans were detectable, however, resolution of the different analogues of hypoxoside and rooperol was not achieved. We focused mainly on hypoxoside and it was interesting to find that the ratio of hypoxoside, dehydroxyhypoxoside and bis-dehydroxyhypoxoside differ markedly in the methanol extracts of *H. rooperi* and *H. latifolia* (Figs. 1 and 2).

The study on the biotransformation of hypoxoside was essential for our research program on the anticancer potential of the molecule. We report several surprising findings. Firstly, neither hypoxoside nor rooperol appear in the blood after oral ingestion of hypoxoside. Secondly, hypoxoside is quantitatively converted to rooperol in the faeces due to bacterial glucosidases in the colon [12]. Thirdly, only phase II metabolites of rooperol are found in the blood. An extremely effective first pass metabolism of rooperol therefore exists. A complete description of the pharmacokinetics of hypoxoside taken by cancer patients in a phase I trial will be presented elsewhere. Of greater relevance to this study, however, is the fact that our analytical HPLC system presented a suitable method to establish that all three rooperol analogues were present in the respective phase II metabolites. Our initial attempts to elucidate the structures of the compounds present in fractions A, B and C (Figs. 4 and 5) by high-resolution mass spectrometry and NMR studies gave confusing results which, retrospectively, were due to the differences in hydroxyl content of the rooperol analogues.

The ratio of the different rooperols generated by enzyme hydrolysis of fractions A, B and C (Fig. 7) differ markedly from that of the ingested hypoxoside which contained mainly rooperol as the aglucone. This might be partly artefactual, since the conversion of hypoxoside analogues from *H. latifolia* by β-glucosidase to rooperol analogues also changed the ratio (Fig. 2) despite the fact that the absorption maxima at 260 nm (Fig. 3C) were not drastically altered. However, the fact that rooperol is the major constituent in faeces (Fig. 5) also suggests that preferential absorption of dehydroxy- and bis-dehydroxyrooperol might occur. This focuses on the importance of which *Hypoxis* species should be used for possible therapeutic application. As shown in Figs. 1 and 2 *H. latifolia* contains a higher proportion of dehydroxy and bis-dehydroxy hypoxosides than *H. rooperi*.

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