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Note

High-performance liquid chromatographic methods for the analysis and purification of quassinoids from *Quassia amara* L.

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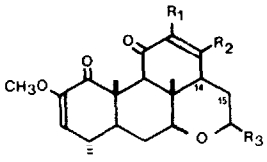
The seco-triterpene quassin (Fig. 1, I) has important pharmaceutical and insecticidal properties[1] and its intensely bitter nature makes it ideal as a bittering agent for beverages and foodstuffs. Crude quassin, extracted from the wood of the neotropical species *Quassia amara* L. and *Picrasma excelsa* (Swartz.) Planch., has been shown to contain quassin, neoquassin (II) and 18-hydroxyquassin (IV)[2].

Class 1



		R_1	R_2	R_3
I	Quassin	OCH_3	CH_3	=O
II	Neoquassin	OCH_3	CH_3	HOH
III	12-hydroxyquassin	OH	CH_3	=O
IV	18-hydroxyquassin	OCH_3	CH_2OH	=O
V	14,15-dehydroquassin	OCH_3	CH_3	=O

Fig. 1. Structures of quassinoids referred to in the text.

As part of our interest in the biosynthesis and biotechnological production of bittering agents we have been led to evaluate the analytical procedures currently available for quassin. Although high-performance liquid chromatographic (HPLC)[3,4], thin-layer chromatographic (TLC)[5,6] and gas chromatographic (GC)[7,8] methods are available these are unsatisfactory, showing incomplete resolution of the compounds present. Enzyme-linked immunosorbent assays of great sensitivity to quassin have been developed in our laboratories[9,10] but a method giving a complete quantitative analysis of all quassinoids present in our extracts is also required and so we have developed the new chromatographic procedure reported here.

The use of a bonded cyanopropyl column in the normal phase using a simple binary solvent system with isocratic elution is described. C_{18}-bonded phases (Spherisorb-ODS and μBondapak) were also examined but, in comparison, gave broad, poorly resolved peaks as observed previously[4]. Furthermore, the non-aqueous solvents used in the normal phase facilitate the recovery of components for identification by chemical analysis.

In addition, in order to raise antibodies to quassin[10], the purification of 18-

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hydroxyquassin from a commercial, partially purified mixture of quassins was required. This was achieved by preparative HPLC on a silica column also using a binary solvent system but with non-linear gradient elution. Fractions were collected and their identity confirmed by nuclear magnetic resonance (NMR) and mass spectrometry (MS). The most rapidly eluted component is shown to be 14,15-dehydroquassin and not isoquassin as previously reported[2].

EXPERIMENTAL

Chloroform, dichloromethane, methanol and acetonitrile were all of AnalaR grade (BDH). Chlorinated solvents were redistilled prior to use. A partially purified quassin extract was obtained from Koch-Light Labs.

Analytical

The liquid chromatograph consisted of a Waters 6000A pumping module, an Anachem oven, a Rheodyne 7125 valve injector (20-μl loop) and a Pye Unicam LC3 variable wavelength UV detector linked to a Spectra-Physics 4100 computing integrator. The column (25 × 0.49 cm I.D.) of Spherisorb S5-CN (Hichrom) was maintained at 27°C and eluted at 0.9 ml min^{-1} with optimal peak detection at 256 nm. The mobile phase contained 0.5% acetonitrile in chloroform and was degassed by sonication *in vacuo* immediately before use. When required, fractions were collected on a Gilson 201 fraction collector. A standard solution of mixed quassins at 0.1 mg ml^{-1} in chloroform was used.

Preparative

A DuPont series 8800 HPLC system fitted with a column (25 × 2.12 cm) of Partisil-5 (HPLC Technology) was used at 35°C with a flow-rate of 10.0 ml min^{-1}. Peaks were detected at 256 nm and collected on a Gilson 201 fraction collector. Partially purified quassin was dissolved at 100 mg ml^{-1} and 1.0 ml injected per run. The column was eluted using a 15 min duration non-linear gradient (pre-programmed gradient number 3) from 0.5–4.5% methanol in chloroform, followed by 5 min at 5% and a 10-min reversed gradient to restore initial conditions.

Components were identified by high resolution ^1H NMR (300 MHz) on a Bruker Pulse NMR spectrometer CXP and by positive-ion fast atom bombardment mass spectrometry (MS) on an AE902 mass spectrometer.

RESULTS AND DISCUSSION

The use of a cyanopropyl bonded phase has greatly improved the resolution with which quassins may be separated by HPLC (Fig. 2). On Partisil-5 (HPLC Technology) in the normal phase peaks 3 and 4 are poorly resolved and peak 5 lies beneath them (Fig. 3). This could not be significantly improved either by the use of gradient elution or by using a dichloromethane–methanol solvent system. Peak shape is also improved by using the CN-bonded phase and the superior resolution achieved has been maintained on four separate columns, two packed in our laboratory. A similar elution is obtained with 0.5% methanol in dichloromethane but the problems caused by solvent gas-bubble formation make this system less satisfactory for routine use.

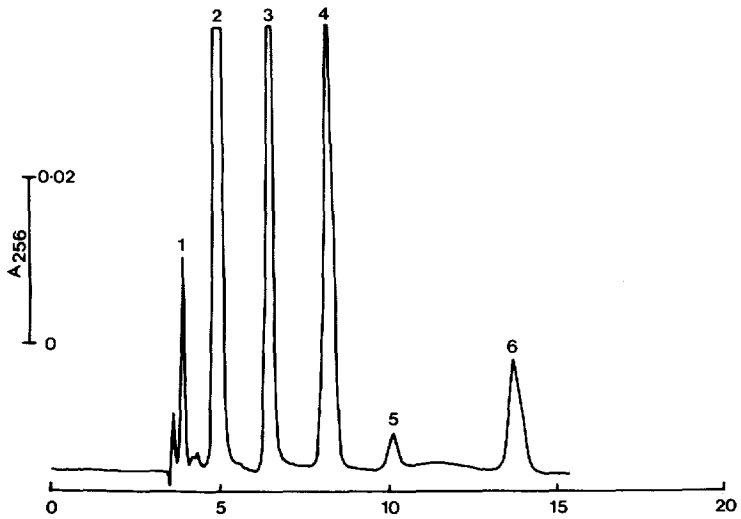

Fig. 2. Separation of a mixture of quassins on a Spherisorb S5-CN column. UV detector at 256 nm. Solvent, 0.5% acetonitrile in chloroform. Flow-rate, 0.9 ml min^{-1}. Temperature, 27°C. Peaks: 1 = 14,15-dehydroquassin, 2 = quassin, 3 = neoquassin, 4 = neoquassin, 5 = 12-hydroxyquassin (see text), 6 = 18-hydroxyquassin.

The chemical identity of the peaks observed by HPLC has previously been assigned by comparison with TLC. Nestler *et al.*[3] only identify quassin and neoquassin, a third component being unidentified. By preparative HPLC we have purified four compounds, peaks 1, 2, 3 + 4 and 6 and identified these as 14,15-dehydroquassin (V), quassin (I), neoquassin (II) and 18-hydroxyquassin (IV) by NMR and MS analysis (Table I). Because of its proximity to quassin peak 1 was repurified

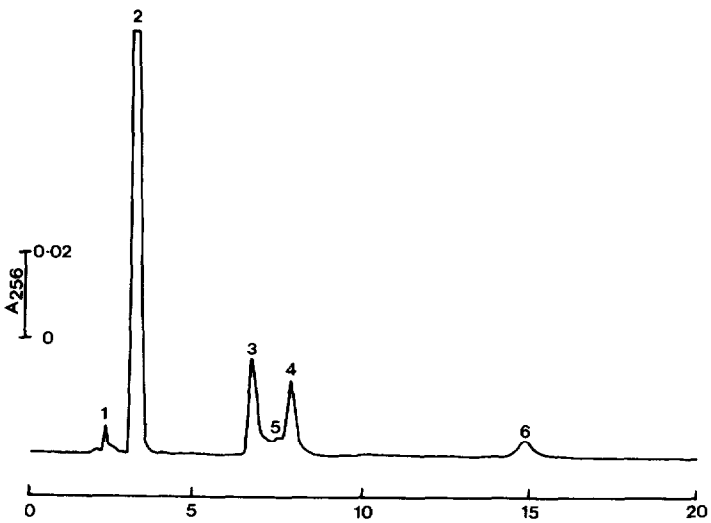

Fig. 3. Separation of a mixture of quassins on a Partisil-5 column. UV detector at 256 nm. Solvent, 1.5% methanol in dichloromethane. Flow-rate, 2.0 ml min^{-1}. Temperature, ambient. Peaks as in Fig. 2.

TABLE I

^{1}H NMR (δ in ppm) AND MS DATA CONFIRMING THE IDENTITY OF PEAKS PURIFIED BY PREPARATIVE HPLC

Values in parentheses are from refs. 2, 11 and 12.

Proton	*1* *14,15-Dehydroquassin*	*2* *Quassin*	*3 + 4* *Neoquassin*	*6* *18-Hydroxyquassin*
C_2OCH_3	3.60 (3.59)	3.60 (3.58)	3.59 (3.59)	3.60 (3.60)
C_3	5.35 (5.39)	5.32 (5.30)	5.27 (5.28)	5.33 (5.32)
C_4	2.47	2.50 (2.40)	2.40 (2.32)	2.50
C_4-CH_3	1.18 (1.10)	1.13 (1.11)	1.08 (1.09)	1.14 (1.13)
C_5	1.87	1.80	n.a.*	1.80
C_6	2.22, 1.96	2.10, 1.90	n.a.	2.11, 1.90
C_7	4.31 (4.28)	4.29 (4.28)	3.95 (3.92)	4.33 (4.38)
C_8-CH_3	1.28 (1.25)	1.21 (1.19)	1.09 (1.08)	1.21 (1.20)
C_9	3.12	3.00 (2.95)	3.19 (3.20)	3.03 (3.02)
C_{10}-CH_3	1.54 (1.52)	1.58 (1.55)	1.54 (1.53)	1.58 (1.56)
C_{12}-OCH_3	3.83 (3.78)	3.68 (3.65)	3.65 (3.65)	3.73 (3.67)
C_{13}-CH_3	2.03 (2.02)	1.89 (1.87)	1.86 (1.85)	–
C_{14}	–	2.40 (2.40)	n.a.	2.79
C_{15}	6.01 (6.03)	3.00, 2.62 (2.67)	n.a., n.a.	3.09, 2.63
C_{16}	–	–	4.79	–
C_{18}	–	–	–	4.56, 4.27 (4.42)
Mass ion (MH^+)	387	389	391	405

* n.a. = not assigned.

by a second passage through the preparative HPLC system. This component runs just ahead of quassin on TLC[2] and has previously been mis-identified as isoquassin, the C-14 epimer of quassin. The NMR spectrum from peak 1 corresponds, however, to that of 14,15-dehydroquassin (Table I) and the molecule shows the correct molecular mass (MH^+ = 387).

Peaks 3 and 4 both show the NMR spectrum (Table I) and molecular mass (MH^+ = 391) of neoquassin and appear to be the 16α and 16β isomers. Confirmation of this is difficult to obtain as isomerisation takes place within a few hours. If, however, the fractions are rapidly dried off *in vacuo* and analysed by an immunoassay they show distinct levels of recognition compatible with this interpretation[9,10].

Too little of peak 5 was isolated by analytical HPLC to identify this component. In an immunoassay it reacts very like quassin[9,10] and is tentatively assigned as 12-hydroxyquassin (III) on the basis of its chromatographic and immunological properties.

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REFERENCES

1 *The Merck Index,* Merck & Co., Rahway, NJ, 1976, 9th ed.
2 C. G. Casinovi, P. Ceccherelli and G. Grandolini, *Ann. Ist. Super. Sanita,* 2 (1966) 414.
3 T. Nestler, G. Tittel and H. Wagner, *Planta Medica,* 38 (1980) 204.
4 F. Tateo and V. Redaelli, *Riv. Soc. Ital. Sci. Aliment.,* 9 (1980) 427.
5 R. Rousselet and R. Paris, *Plantes Med. Phytother.,* 5 (1971) 159.
6 H. Wagner, S. Bladt and K. Münzing-Vasiram, *Pharm. Z.,* 120 (1975) 1262.
7 A. M. Humphrey, in D. G. Land and H. E. Nurston, (Editors), *Progress in Food Flavour Research,* Applied Science Publishers, London, 1979, p. 99.
8 G.Grandolini, M. C. Tiralti and R. Dommarco, *Riv. Soc. Ital. Sci. Aliment.,* 9 (1980) 277.
9 R. J. Robins, M. R. A. Morgan, M. J. C. Rhodes and J. M. Furze, *Anal. Biochem.,* 135 (1983) in press.
10 R. J. Robins, M. R. A. Morgan, J. M. Furze and M. J. C. Rhodes, *Phytochemistry,* in press.
11 Z. Valenta, S. Papadopoulos and C. Podesva, *Tetrahedron,* 15 (1961) 100.
12 R. M. Carman and A. D. Ward, *Austr. J. Chem.,* 15 (1962) 807.