

Stability-indicating LC assay of and impurity identification in homoharringtonine samples[☆]

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Abstract

Homoharringtonine (HHT) is a potent myelosuppressive agent and has antitumor activity. Recent studies suggest that it inhibits tumor growth by inducing apoptosis. HHT is an ester of the alkaloid cephalotaxine. It is isolated from genus *Cephalotaxus*. At least ten HHT analogs have been identified from cephalotaxus extracts. High performance liquid chromatography (HPLC) separations of the cephalotaxine alkaloids in plant extracts have been reported, they have not been validated as specific and stability-indicating for HHT. Due to the complexity of the alkaloid extracts, it is conceivable that additional analogs may still be unresolved from HHT. This paper presents an improved and validated HPLC assay for HHT. The assay is stability-indicating, precise (R.S.D. < 1%), linear ($r^2 = 0.9999$), and accurate (error < 1%). The assay reveals three congeners present as impurities in HHT samples. Two are new and have not been previously reported. Identities of the impurities and forced decomposition products, elucidated with their HPLC retention and spectral data, are also presented. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Homoharringtonine; New congeners; Decomposition products; Identity; Validated stability-indicating HPLC assay

1. Introduction

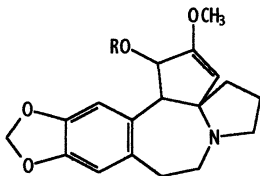
Homoharringtonine (Fig. 1) (**7**, HHT), an alkaloid isolated from genus *Cephalotaxus* [1], is an alkyl substituted succinic acid ester of cephalotaxine (**1**). It possesses antileukemic activity [2–7]

and is a potent myelosuppressive agent [8,9]. Recent studies suggest that HHT inhibits tumor growth by inducing apoptosis [10,11]. Its potent antileukemic activity and limited toxicity have prompted the National Cancer Institute to develop HHT as an antitumor agent. Though synthesis of cephalotaxine and its esters has been reported [12,13], extraction from plants is still the major source for HHT. Ten HHT analogs (**2–11**, Fig. 1), where the substitution on the succinyl moiety varies, have thus far been identified from cephalotaxus extracts [14,15]. Three additional

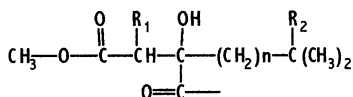
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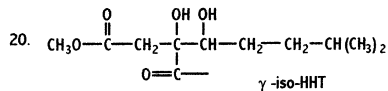
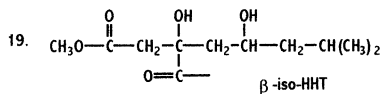
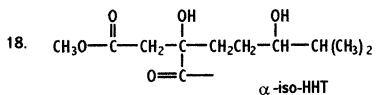
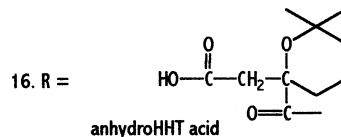
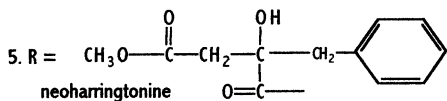
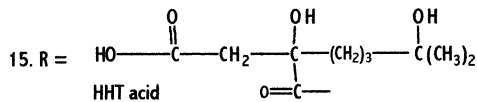
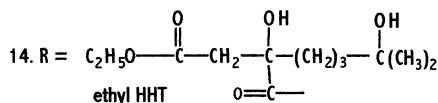
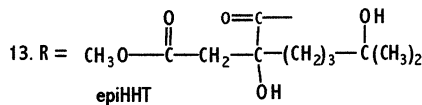
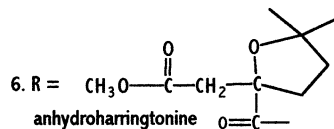
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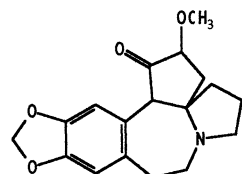
1. R = H; Cephalotaxine



	R ₁	R ₂	n	
2.	H	OH	2	harringtonine (HT)
3.	OH	H	2	isoharringtonine (isoHT)
4.	H	H	2	deoxyharringtonine
7.	H	OH	3	homoharringtonine (HHT)
8.	H	H	3	homodeoxyharringtonine
9.	H	OH	1	norharringtonine
10.	H	H	1	nordeoxyharringtonine
11.	H	H	4	bishomodeoxyharringtonine
12.	OH	H	3	isoHHT



17.



tautomer of cephalotaxine

Fig. 1. Structure of homoharringtonine congeners and decomposition products.

congeners, where the cephalotaxine ring is oxygenated, were also isolated [16]. HPLC separations to detect and determine major HHT congeners in *Cephalotaxus* have been reported [17–19]. A high performance liquid chromatography (HPLC) assay for HHT injectables has also been recently reported, however, its stability-indicating capability was not demonstrated [20]. Due to the complexity of the alkaloid extracts, it is conceivable that closely related analogs may still be unresolved from HHT. Because of the current pharmaceutical interest, a stability-indicating HPLC assay specific for HHT analysis is sought.

For over 15 years, the authors' laboratory has been involved in the analysis of HHT samples. The HPLC assay of HHT has been carried out with ODS columns using a mixture of MeOH and H₂O containing 1% conc. NH₄OH (50:45, v/v) as the mobile phase. The assay, though unpublished, has been validated as stability-indicating and is similar to more recently published HPLC separation of HHT [17–20]. The use of NH₄OH in the mobile phase, which resulted in a pH 10.5 for the H₂O solution, was to curtail tailing and improve the shape of the HHT peak, unfortunately, it also caused premature deterioration of ODS columns. In an effort to improve on the column life, the HPLC assay was modified to reduce the alkalinity in the mobile phase. Substituting acetonitrile (ACN) for MeOH improves separation and resulted in the HPLC assay described in Section 2.3. This improved assay revealed an additional impurity that was buried in the tail of the HHT peak in the old method. Since HHT is formulated as lyophilized powder and administered intravenously as saline solutions, the new and improved HPLC assay was validated for stability-indicating with respect to hydrolysis and thermal decompositions.

This paper presents the improved stability-indicating and validated HPLC assay for HHT. The assay revealed three small impurities common to commercial HHT samples. The impurities were identified as HHT congeners by their HPLC retention and spectral data. Two of them are new and have not been previously reported. Identities of forced decomposition products of HHT, deduced from their HPLC retention and mass spectral data, are also reported.

2. Experimental

2.1. Reagents and materials

Glacial acetic acid and ammonium hydroxide were purchased from Mallinckrodt (Paris, KY). Triethylamine (TEA) was obtained from Aldrich–Sigma (St Louis, MO). The chemicals were reagent grade. HPLC-grade acetonitrile (ACN) and methanol (MeOH) were from Burdick & Jackson Division, Baxter Healthcare Corp. (Muskegon, MI). Acid and base solutions were prepared with water purified through a Millipore Super-Q Pure Water System (Waltham, MA). Solutions of HCl and NaOH were prepared from Dilute-it Analytical Concentrate from J.T. Baker (Phillipsburg, NJ).

Homoharringtonine and harringtonine samples were received from the National Cancer Institute (Bethesda, MD). An impurity enriched HHT sample, obtained from the mother liquor of recrystallization of a HHT sample, was also received for impurity isolation. Cephalotaxine was purchased from Aldrich–Sigma. The internal standard, *o*-nitroaniline, was purchased from Aldrich (Milwaukee, MI). Internal standard solution (ISS) was prepared by dissolving 10 mg *o*-nitroaniline per 100 ml ACN. Test solutions for assay validation were prepared in ISS to yield solutions of 1–3 mg/ml.

2.2. Forced HHT decomposition

To test the stability-indicating capability of the LC assay, potential hydrolytic and thermal decomposition products of HHT were generated as follows. Aliquots of stock solution of HHT in ACN (2 mg/ml) were diluted with equal volume of ACN, H₂O, acid (1.0 and 0.1 N HCl) or base (0.1 M NH₄OH), respectively, to form solutions S1, S2, S3a, S3b, or S4. The solutions were heated for 2 h in a H2025 Temp Blok Module Heater (Scientific Products, Division of American Hospital Supply, McGaw Park, IL), set at 75°C, to create hydrolysis products. Solid sample of HHT was in a 60°C oven with 60% relative humidity for 30 days to create thermal decomposition (S5).

2.3. HPLC assay

HPLC was performed with a HP-1050 system (Hewlett–Packard, Wilmington, DE). Data were collected and processed with the HP 3D ChemStation. LC-MS was carried out with a Vestec 201 XL mass spectrometer equipped with a thermospray interface (Houston, TX). Ammonium acetate was used in place of TEA-HOAc in the mobile phase to produce ions in the filament-off mode. Source temperature was 300°C and probe tip temperature 240°C. Spectra were scanned from m/z 90 to 700 at 4 s/scan.

Test solutions (10 μ l) were automatically loaded on a Phenomenex (Torrance, CA) IB-SIL C18, 5 μ m, 250 \times 4.6 mm I.D. stainless-steel column. Chromatographic analysis was carried out by isocratic elution, with 24% ACN pump mixed with 76% H₂O containing 0.2% HOAc adjusted to pH 6.5 with TEA (\sim 0.5% TEA), at 1.0 ml/min. The solvent composition % is by volume. Detection was by UV at 280 nm, photodiode array (PDA), or LC-MS. Impurity quantitation was by peak area, assuming equal detection response.

2.4. Impurity isolation

An impurity enriched HHT sample (lot KS22-132-3) was used for impurity isolation. To isolate the impurity, the HPLC conditions were modified as follows. HHT solutions (10 μ l, 10 mg/ml in acetonitrile) were loaded on two Phenomenex (Torrance, CA) IB-SIL C18, 5 μ m, 250 \times 4.6 mm I.D. stainless-steel column connected in tandem. Chromatographic elution was carried out isocratically with 30% ACN pump mixed with 70% 0.1 M ammonium trifluoroacetate buffer, (pH adjusted to between 6.5 with NH₄OH), at 1.0 ml/min. The solvent composition % is by volume. Detection was by UV at 280 nm. Elution fractions corresponding to the target impurity from 300 HPLC runs were collected and pooled. The pooled collection was reduced in volume by a SpeedVac SCV200 centrifuge vacuum condenser (Savant Instrument, Holbrook, NY) to a near dry residue. The residue was redissolved in H₂O and pass

through a Sep-Pak C18 cartridge (Waters, Milford, MA) to remove the ammonium trifluoroacetate. The cartridge was rinsed with 1.0-ml of H₂O, and the retained impurity was eluted with 2 ml ACN. The ACN eluate was then evaporated by SpeedVac condenser, resulting in isolated impurity, which was subjected to HPLC, MS, and NMR analysis.

MS were obtained by electron ionization (EI) using a Ribermag R10-10C mass spectrometer. NMR was obtained with a Gemini 300 (300 MHz) NMR spectrometer from Varian (Palo Alto, CA).

3. Results and discussion

Fig. 2 is the chromatogram of a freshly prepared ACN solution of HHT (S1). In addition to the major peak (a) for HHT, three tiny impurities (b–d) are present. Table 1 lists the amount of impurities in several HHT samples. None of the impurities were enhanced during forced decomposition (Section 2.2). Chromatograms from thermally heated bulk sample (S5) are invariant to that of S1, indicating that bulk HHT is stable. HHT is also stable in neutral solutions. Heating S1 and S2 for 2 h at 75°C did not alter their HPLC profiles. However, heating the acid solutions S3a and S3b similarly generated four products (e, g–i, Fig. 3) while heating the basic solution S4 gave rise to primarily e and a small amount of f (Fig. 4). In strong alkali (0.1 N NaOH), HHT decomposed readily to e and ultimately f (data not shown). Increasing or decreasing the organic modifier (ACN) content in the mobile phase did not reveal additional impurities or significant decomposition products. UV spectra of peaks a–i, obtained from PDA analysis, are identical to each other (Fig. 5), suggesting that their chromophores (the cephalotaxine moiety) are likely identical. This assertion is supported by LC-MS. Mass spectra of HHT (a), its impurities (b–d) and decomposition products (f–i) are presented in Fig. 6. They all have significant fragments of m/e 316 for the cephalotaxine moiety (Fig. 7).

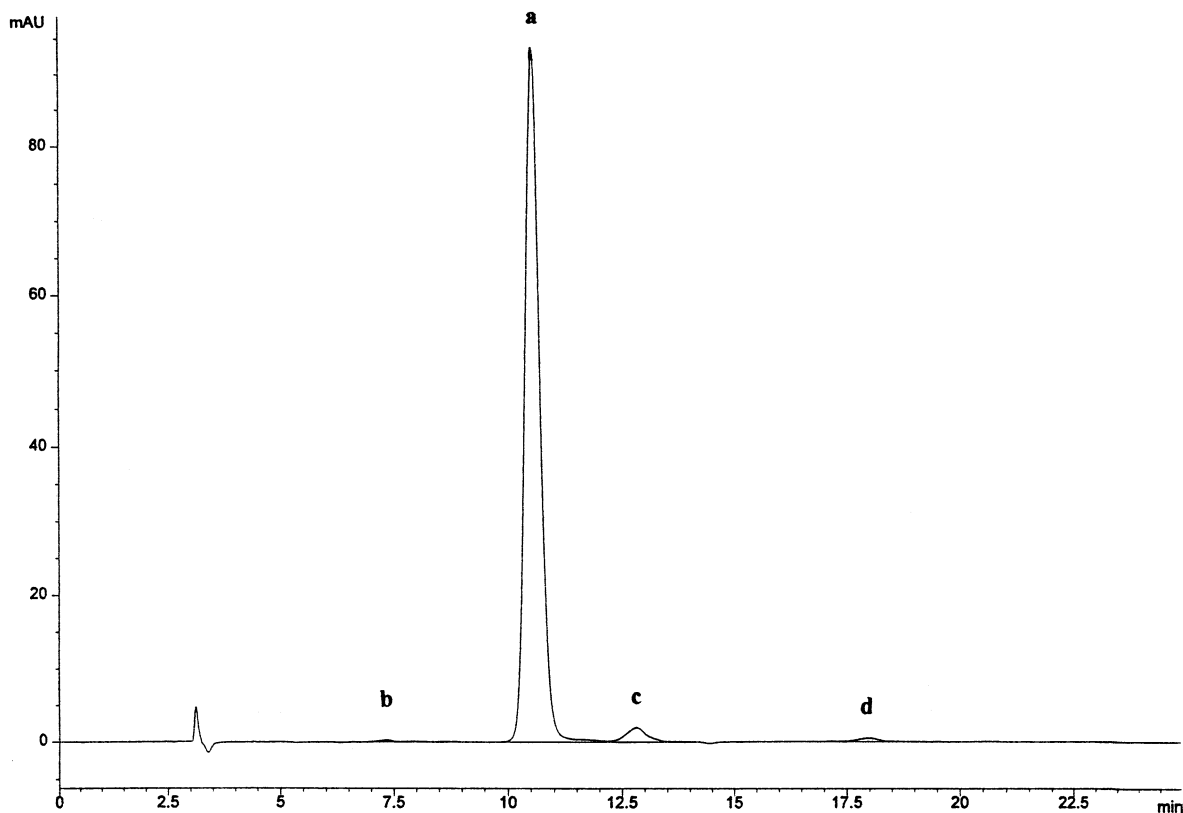


Fig. 2. HPLC chromatogram of a fresh ACN solution of HHT (S1). See Section 2 for details.

3.1. Impurities of HHT

Impurity **b** has pseudomolecular ion ($M + H$) and daughter ion ($D = M - CH_2COOR$, where $R = CH_3$) of 532 and 458, respectively. They are both 14 units less than those of HHT (546 and 472) and suggest that **b** has the same R (CH_3) but one less CH_2 in the alkyl chain as compared to HHT. A likely candidate for **b** is harringtonine (HT). That **b** is HT was confirmed by its coelution with authentic HT standard in HPLC.

Impurity **d** has a $M + H$ of 560, which is 14 units or a methylene (CH_2) more than that in HHT. The 316, 298, and 284 fragments indicate that the extra CH_2 is not in the cephalotaxine moiety. Lack of fragment 486 and presence of significant fragment 472 for the D ion (Fig. 8) suggest that the extra CH_2 in **d** must be in the R of the $-CH_2COOR$ moiety. Hence, **d** must be the ethyl ester analog of HHT. Indeed, **d** was signifi-

cantly enhanced when an ethanol solution of HHT was added with a slight amount of NaOEt. This confirms that **d** is the ethyl ester analog of

Table 1
HPLC impurities in commercial homoharringtonine samples

Lot no. ^a	% Impurity ^b		
	b	c	d
800528	0.1	1.3	–
871203	–	1.0	–
921115	0.3	3.0	0.8
960625	–	1.8	0.3
800722	0.1	0.9	0.2
KS-22-130-2	–	1.5	0.9
KS-22-132-3 ^c	1.8	10.7	0.7

^a Supplied by the National Cancer Institute, NIH, USA.

^b Normalized peak area, assuming equal detection response.

^c From mother liquor of purification of lot 921115. This lot was used to isolate impurity **c**.

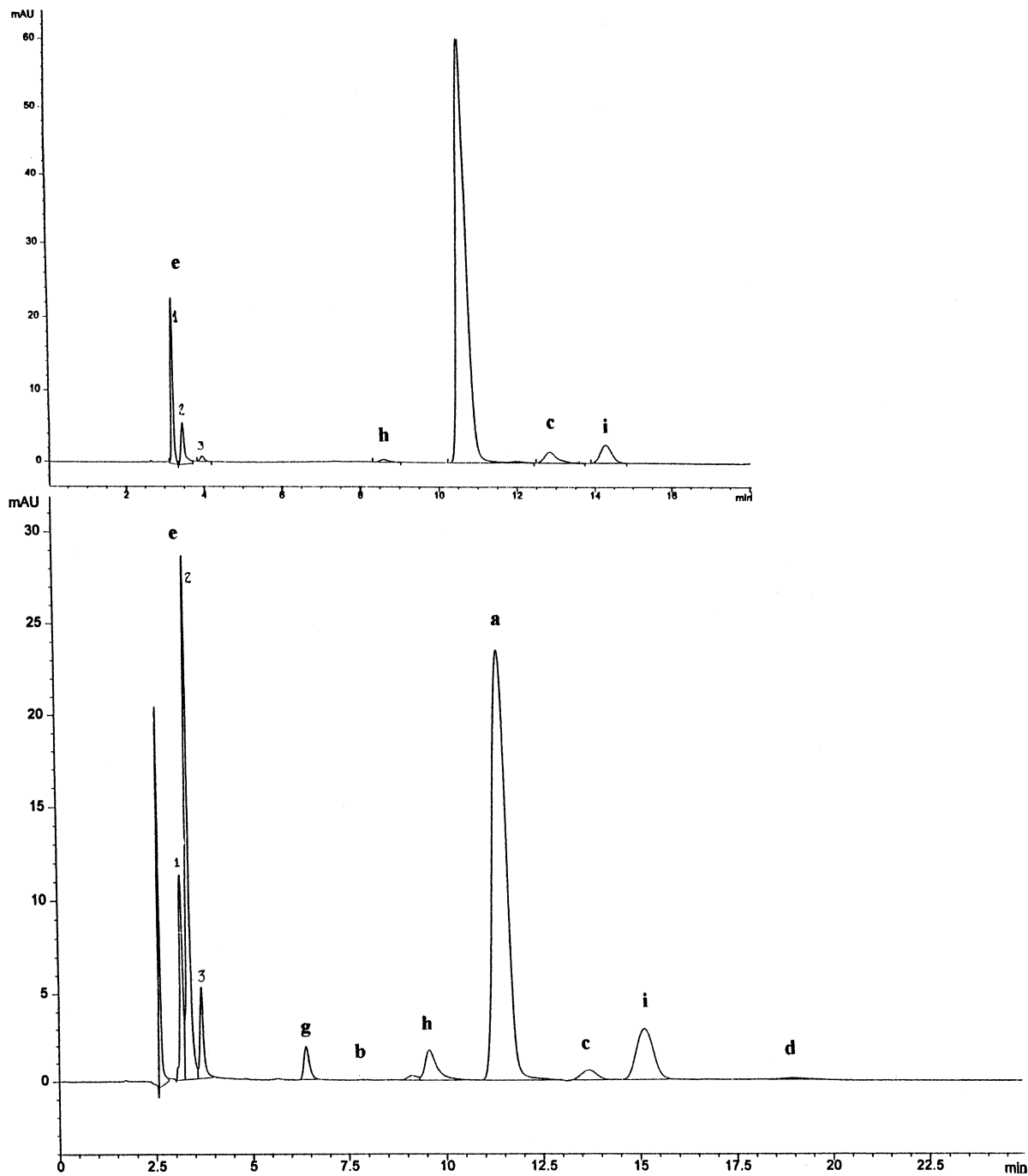


Fig. 3. HPLC chromatogram of acid solutions of HHT (S3), heated for 2 h at 75°C. (top) 0.1 N HCl, (bottom) 1.0 N HCl. Peak e exists in three ionic states.

HHT (14). Impurity **d** may be an impurity resulted from transesterification during the extraction/purification process, since EtOAc was used in the process [21].

The LC-MS of impurity **c** is identical to that of HHT(**a**), suggesting that the two are isomeric to each other. To further characterize **c**, it was isolated by HPLC from the impurity enriched HHT sample as described in Section 2.4. The isolated material was subjected to HPLC, LC-MS, direct EI-MS and NMR experiments.

When a 0.5-mg/ml solution of the isolated material in 0.1 N NaOH-ACN (1:1) was heated at 80°C for 2 h, the major product formed is identical to authentic cephalotaxine in HPLC retention and in LC-UV and LC-MS data. This suggested that the cephalotaxine moiety is not altered in **c**.

EI-MS of a trimethylsilylated samples of HHT and the isolated **c** indicate that TMS derivative was not formed from HHT while a mono-TMS

derivative was formed from **c**. This information suggests that **c** has a non-tertiary –OH group in the alkyl side chain. In the $^1\text{H-NMR}$, the 1.2-ppm singlet for the terminal dimethyl group of HHT (Fig. 8, bottom) is seen replaced by a pair of doublets at 0.85 ppm in **c** (Fig. 8, top). The multiplicity (doublets with $J = 7$ cps each), chemical shift (0.85 ppm), and relative intensity (three protons each) of the 0.85 doublets are consistent with a $-\text{CCH}(\text{CH}_3)_2$ group where the two $-\text{CH}_3$ are slightly non-equivalent. In addition, the spectrum for **c** shows new multiplets at 1.5 and 3.3 ppm (indicated with arrows in Fig. 8, top), each accounts for one proton and are absent in the spectrum for HHT. This is consistent with the presence of two methine protons in **c**, one is shielded by an electron rich group such as an –OH. These accumulative information confirm that the isopropyl OH group in HHT migrated away from the terminal isopropyl carbon in **c**.

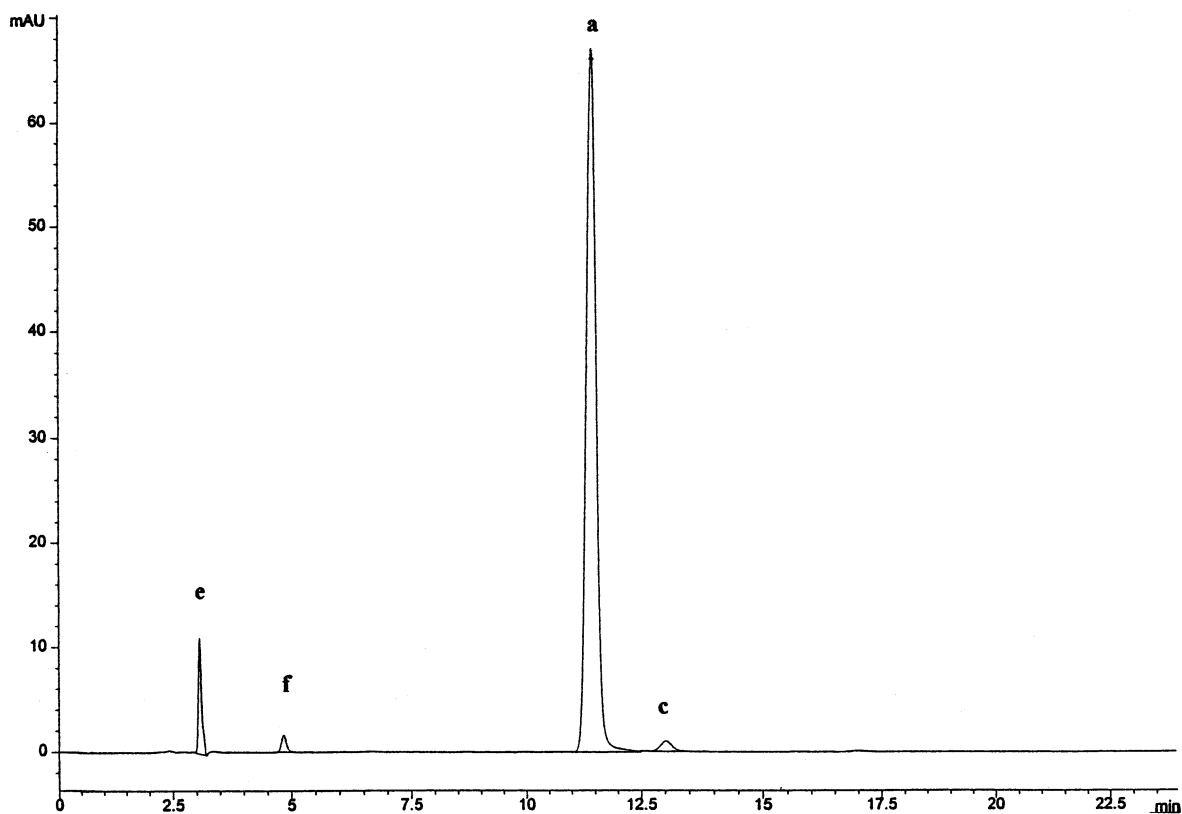


Fig. 4. HPLC chromatogram of a basic solution of HHT (S4), heated for 2 h at 75°C. See Section 2 for details.

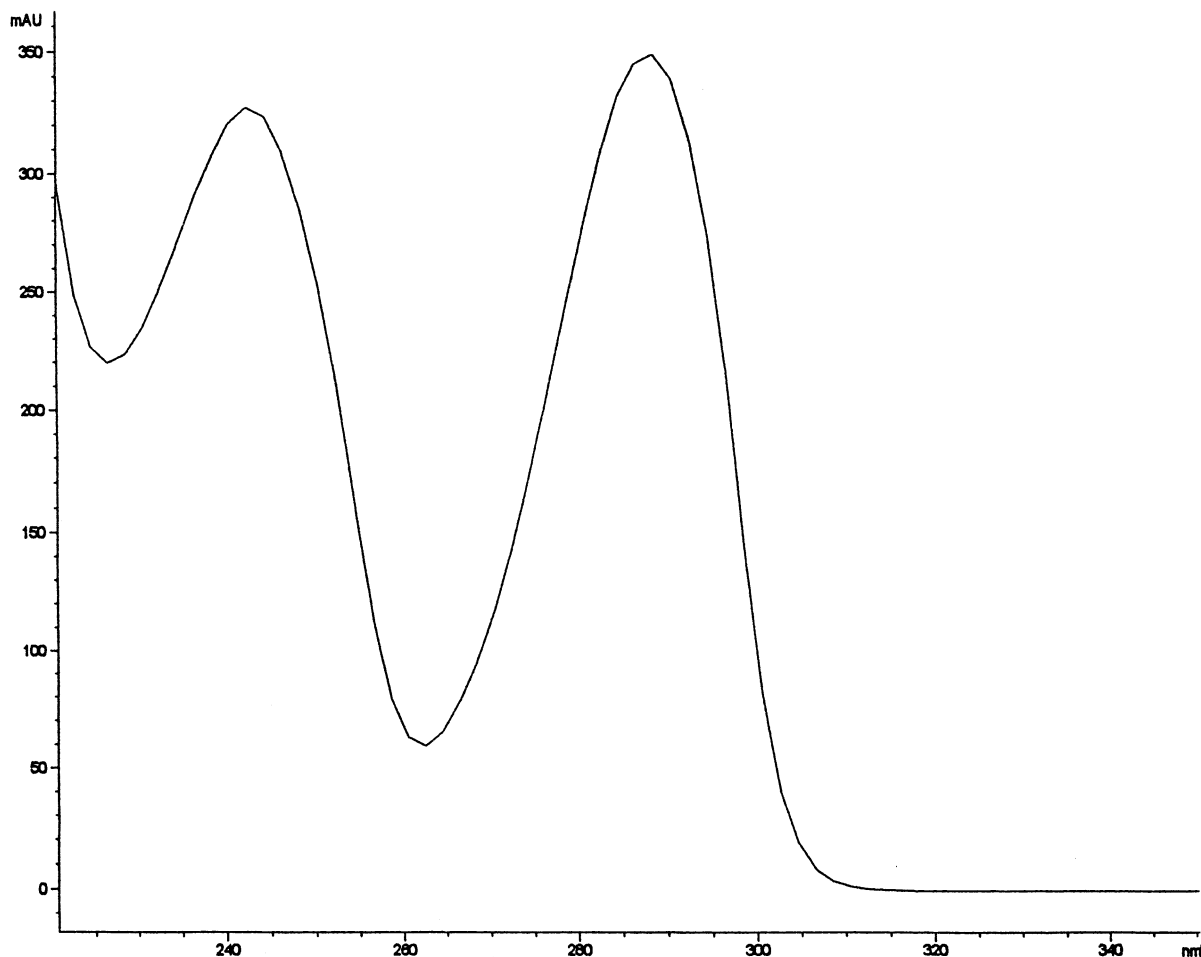


Fig. 5. UV spectrum of HHT detected by PDA. Spectra for the impurities and decomposition products are identical.

Possible candidates for **c** are isoHHT(**12**), and other positional isomers involving the OHs on the alkyl side chain.

The possibility of **c** as isoHHT is being ruled out by MS and HPLC considerations, and NMR signals. IsoHHT would have D ion 16 mass units less than observed for **c** (i.e. isoHHT should have a fragment from $M\text{-CH}_3\text{O-CO-CH(OH)-} = 456$ instead of a fragment from $M\text{-CH}_3\text{-CO-CH}_2\text{-} = 472$ in Fig. 6c). In HPLC conditions similar to this paper, retention of isoHT(**3**) doubles that of HT(**2**) [17,18]. Apparently, moving the OH from the alkyl terminal in HT to the middle of the succinate resulted in a much more lipophilic isopentane side chain for isoHT. Analogously,

isoHHT would be expected to be much more retained than HHT. Since the relative retention of **c** is 1.2 of that of HHT, **c** is unlikely to be isoHHT. Besides, the doublet pair at 1.95 and 2.28 ppm for the $\text{-OCOCH}_2\text{C(OH)-}$ methylene protons in HHT (marked a in Fig. 8, bottom) remains unaffected in **c** (Fig. 8, top, though the 1.95 ppm pair was covered by the residue ammonium signal) and confirms that **c** is not isoHHT.

Therefore, **c** is narrowed to one of the three isomers of HHT with the -OH group sitting on one of the three methylene carbon on the isohexane side chain (**18**, **19**, **20**). A $^1\text{H-COSY}$ NMR would further pin down the position of the OH. A connection of the 0.85- and 1.5-ppm signals will

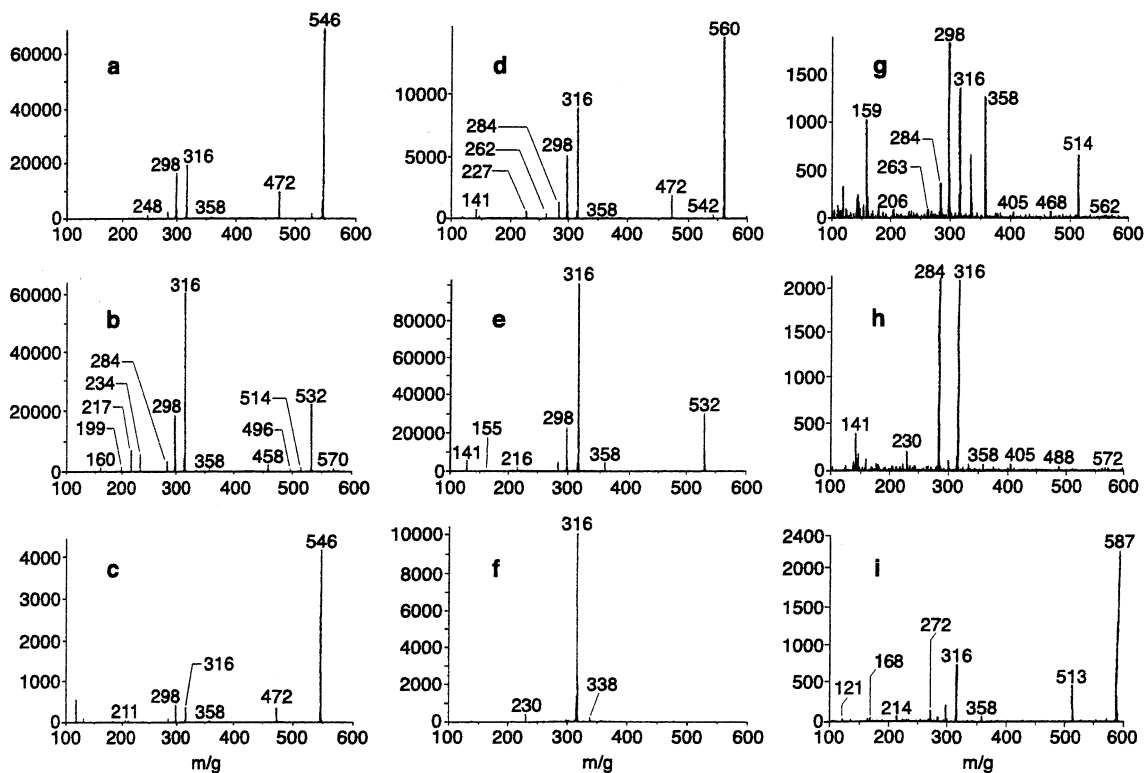


Fig. 6. LC-MS of peaks a–i.

be expected in all three possible structures. Only in the α -*iso*-HHT (**18**) the connectivity of the 1.5- and 3.3-ppm signal would be expected, which is indeed observed in the COSY-NMR of **c** (Fig. 9). This confirms that **c** is the α -isomer of HHT (**18**), an *iso*-homoharringtonine, in which the –OH sits on the carbon atom α to the isopropyl terminal.

3.2. Decomposition products of HHT

The major acid decomposed products (Fig. 3, **e1–e3**) and the major product from the basic solution (Fig. 4, **e**) have identical MS (a representative spectrum is presented as Fig. 6e). Their generation, lack of HPLC retention, $M + H$ (532), and multi-speciation are consistent with them being the carboxylic acid (**15**) of HHT. The free acid was formed from hydrolysis of the methyl ester in the side chain, resulting in a $M + H$ 14 mass units less than that of the parent (HHT, 546). The three peaks **e1–e3** in Fig. 3, from the acidic solutions, represent three ionic species of **15** involving the

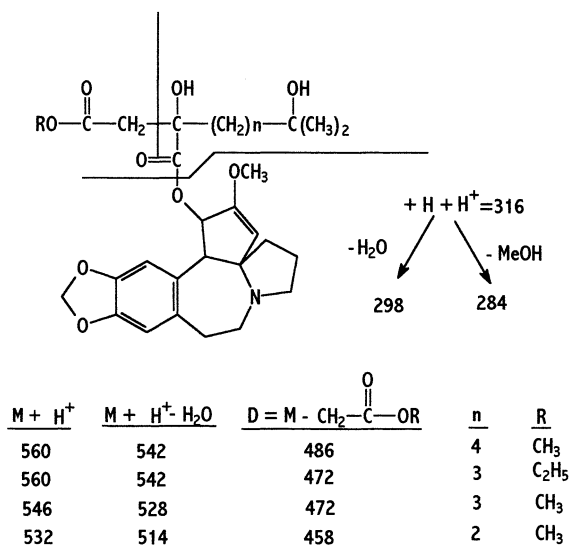


Fig. 7. MS fragmentation pattern of HHT and analogs.

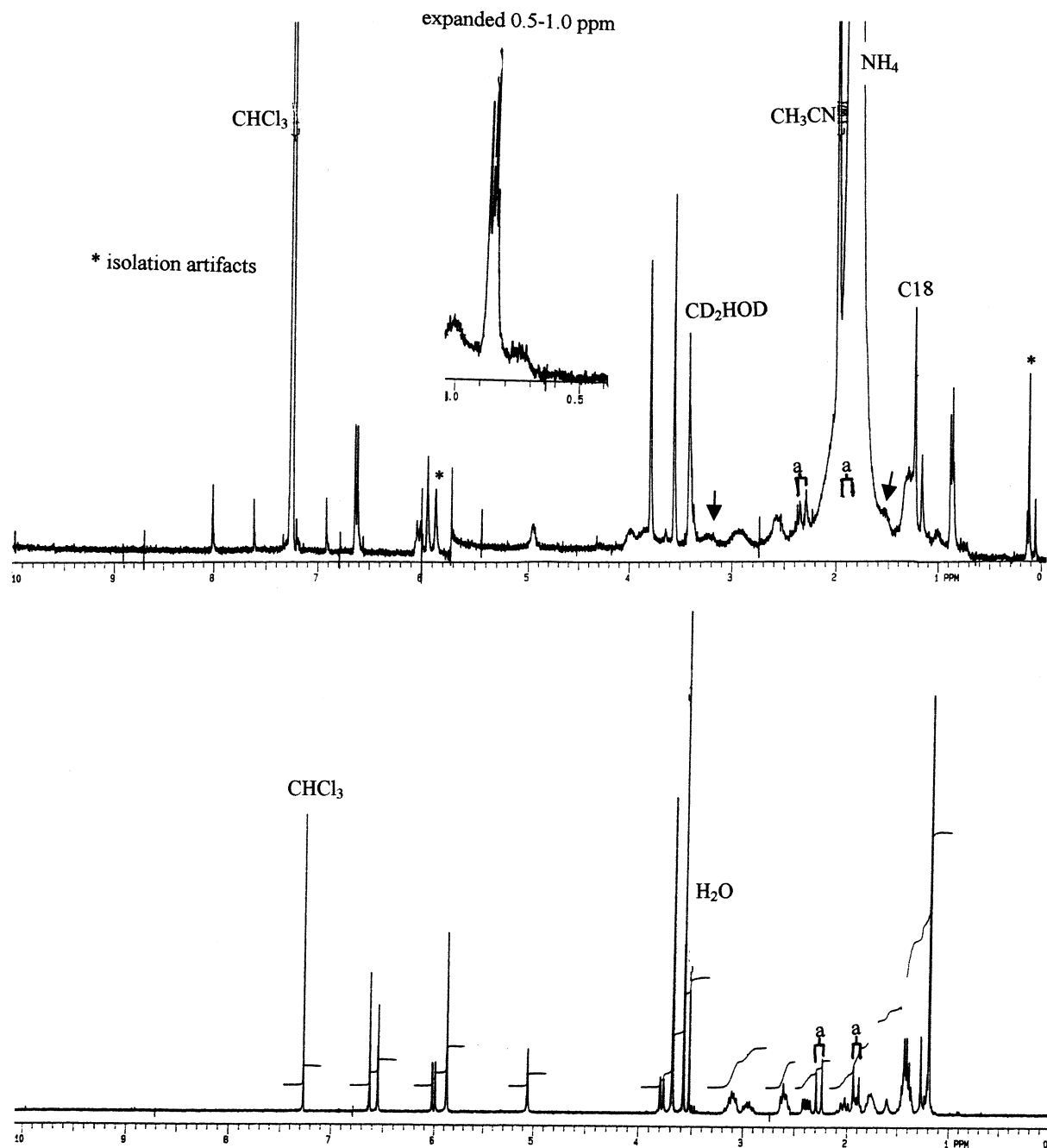


Fig. 8. Proton NMR spectra of (top) impurity **e** isolated by HPLC, ~ 0.5 mg/ml CDCl_3 , and (bottom) a HHT sample, 10 mg/ml CDCl_3 . Spectra were obtained with a Varian Gemini 300 NMR spectrometer.

ionization of the amine and carboxylic acid groups. When the acid solution was diluted (1:3) with the aqueous part of the mobile phase, only a single

unretained peak (**e2**, $k' \sim 0.1$) was observed. Contrary to the MS of the esters (Fig. 6a–d) there is no detectable daughter ion D for the free acid **e**.

Product **g** has a $M + H$ of 514 and fragments of 316 and 298. The latter two indicate the presence of intact cephalotaxine. The $M + H$ of 514 and the lack of the corresponding D ion (440) suggest that **g** could be the free acid of a dehydro-HHT or anhydro-HHT (**16**). This suggestion is consistent with the relative HPLC retention of **g** to **e**. In some mildly acid decomposed solutions of HHT (data not shown), a tiny and very late eluting product ($k' \sim 27$) was formed. The MS of the product ($M + H = 528$, $D = 454$, 316, 298) is con-

sistent with the methyl ester of **g**, the dehydro- or anhydro-HHT. The k' ratio of anhydro-HHT to HHT is 10 and is similar to that of **g** (**16**) to **e** (**15**). MS of product **h** has a molecular ion of 316 and a prominent fragment of 284. This suggests that **h** is a cephalotaxine analog with an easy leaving $-\text{OCH}_3$ group. A likely candidate for **h** is the cephalotaxine tautomer **17**.

Product **i** has a $M + H$ of 587 and is believed to be the acetonitrile adduct of HHT (this product can only be generated when HHT was heated in

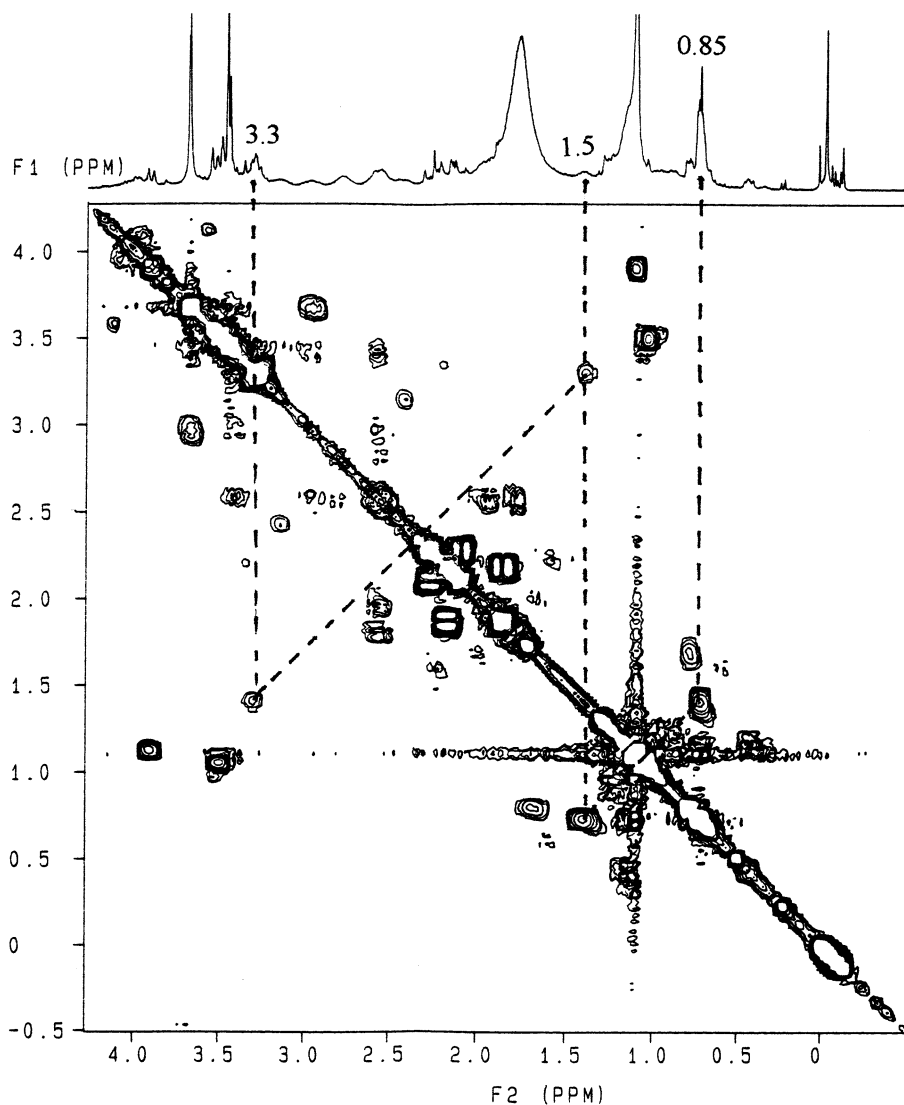


Fig. 9. ^1H -COSY NMR of isolated impurity **c**.

Table 2
HPLC retention (k'), mass spectral data, and identities of HPLC peaks in HHT samples

Peak	k'	MS data ^a	Identity
a	2.7	546(M+H), 472(D), 316, 298, 284	HHT (7)
b	1.5	532(M+H), 458(D), 316, 298, 284	HT (2)
c	3.5	546(M+H), 472(D), 316, 298	HHT isomer (18)
d	5.2	560(M+H), 472(D), 316, 298, 284	Ethyl-HHT (14)
e	0.1–0.3	532(M+H), 316, 298, 284	HHT acid (15)
f	0.5	316(M+H), 298	Cephalotaxine (1)
g	1.1	514(M+H), 358, 334, 316, 298, 284	Anhydro-HHT acid (16) ^b
h	2.0	316(M+H), 284	Cephalotaxine tautomer (17) ^b
i	4.0	587(M+H), 513(D), 316, 298	HHT-ACN adduct

^a A small fragment (358), present in some spectra more significantly than in others, is the acetylcephalotaxine generated during the MS process.

^b Identity is tentative.

an acetonitrile solution but cannot be generated in a methanol solution). Presence of the D (513) and the 272 (succinyl side chain + ACN) fragments suggest that the adduct occurs at the dihydroxyhexyl portion, but the exact position and structure of this product is unknown. The base product **f**, formed slowly in mildly basic media but easily in strong alkali is cephalotaxine (**1**). Its identity was confirmed by its MS ($M + H = 316$, $M + Na = 338$) and by its coelution with authentic cephalotaxine in HPLC.

Table 2 summarizes the identity, HPLC retention and mass fragmentation pattern of HHT, its impurities and hydrolysis products. The identities for **g** and **h**, however, are tentative and have not been fully proofed.

3.3. HPLC assay and validation

Fig. 10 is the HPLC chromatogram of a test solution for the HHT assay. The test solutions were prepared by dissolving 1-mg portions of HHT samples in 1.00 ml aliquots of the internal standard (IS) solution (10 mg *o*-nitroaniline in 100 ml ACN). Results were calculated from the peak area ratio of HHT/IS. Precision of the HPLC assay is 0.5% ($n = 6$) within-day and 0.7% between days ($n = 4$). Based on data (Table 3) from five standard solutions the assay was linear ($r^2 = 0.9999$) and accurate (0.4% error). The accuracy of the method was also demonstrated by the analysis of two lots of HHT sample. The purities

were established as 97.0% for lot KS22-130-3 and 98.5% for lot 800528, respectively, by a material balance consideration of impurities, moisture, organic volatile, and residue on ignition. The LC assay for lot KS22-130-2, using the 98.5% pure lot 800528 as the working standard, is 97.3% ($s = 0.5$, $n = 5$) which is within experimental error of that derived from mass balance consideration (97.0%). The assay has a minimum linear range of 4–12 μg of HHT and the limit of quantitation (LOQ) was 27 ng.

4. Conclusions

An improved stability-indicating HPLC assay for HHT has been developed and validated. The assay is precise, linear and accurate. Identities of impurities and some forced decomposition products have been elucidated. Identities of other decomposition products have been suggested. Of the three impurities, one is harringtonine, one is an isomer of HHT with the $-\text{OH}$ moved to the carbon atom α to the isopropyl terminal, and the other is the ethyl ester analog of HHT. The latter two have not been previously reported.

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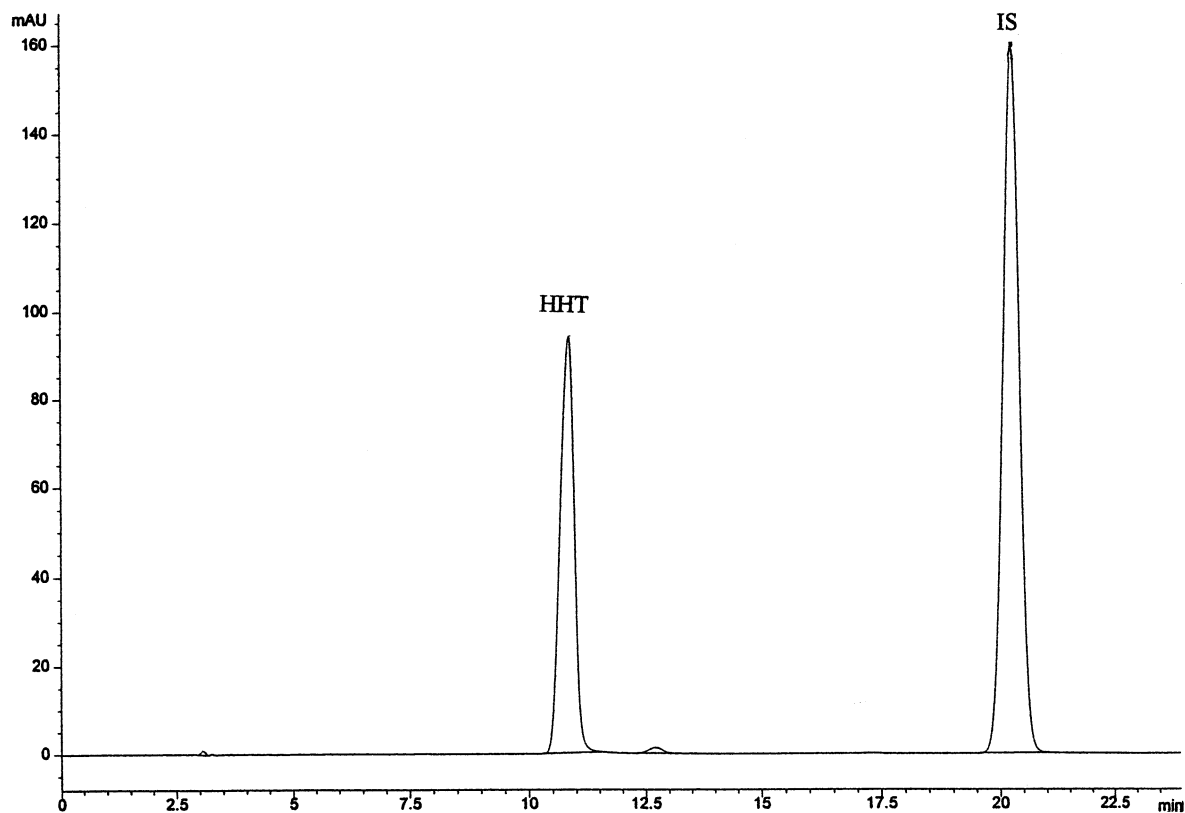


Fig. 10. Typical HPLC chromatogram for HHT assay. See text for the HPLC condition.

Table 3
Linearity and accuracy of the HPLC assay for HHT^a

Sample	Peak intensity			HHT conc. (mg/ml)		Error ^b (%)
	HHT	IS	F = HHT/IS	Actual	Found ^c	
1	9332	19944	0.4679	0.3938	0.3968	+0.75
2	13411	19940	0.6726	0.5719	0.5662	-0.99
3	19410	20158	0.9629	0.8044	0.8066	+0.28
4	21783	19944	1.0922	0.9126	0.9137	+0.12
5	28489	20028	1.4225	1.1878	1.1872	-0.05
Avg ^d						0.44

^a See text for HPLC conditions. Linear regression analysis of $F(x)$ vs actual HHT concentration (y) gave $y = 1.2076x - 0.0112$, $r^2 = 0.9999$.

^b Error = $100 \times (\text{found HHT conc.} - \text{actual HHT conc.}) / \text{actual HHT conc.}$

^c Found HHT concentration = $(F + 0.0112) / 1.2076$.

^d Avg = $\Sigma|\text{error}|/5$.

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