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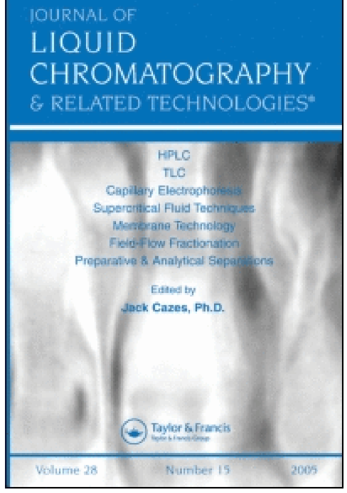

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High Pressure Liquid Chromato-Graphic Separation of Aryl-Naphthalide Lignan Lactones

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HIGH PRESSURE LIQUID CHROMATOGRAPHIC SEPARATION OF ARYLNAPHTHALIDE LIGNAN LACTONES

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ABSTRACT

A high-pressure liquid chromatographic system is described for the separation of few arylnaphthalide lignan lactones. An octadecyl-silica (Spherisorb 5 ODS) column was used with methanol/water (73/27 volume %) isocratic system as eluent. Both HPLC-fluorometry and HPLC-UV detection methods were employed. The present technique is compared with other instrumental methods developed earlier and its utility in chemotaxonomic and toxicologic studies is discussed.

INTRODUCTION

Arylnaphthalide lignan lactones are naturally occurring dimers in plants and are of interest to chemists and medical scientists because of their possible potential use as anti-cancer agents (1,2).

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A few of them find application in industry as antioxidants and insecticides. A few others possess therapeutic as well as toxic properties (3) and find use as suicidal and homicidal agents-hence of interest to clinical and forensic toxicologists.

Cleistanthus collinus (Roxb.) Benth. & Hook. a Euphorbiacean shrub, found widely distributed in tropical countries, is a highly poisonous plant. Though all parts of it are reported to be toxic, a decoction of the crushed leaves is mainly used as suicidal and cattle poison and for procuring criminal abortion (4,5). Chemical characterization of different parts of the plant has led to the identification and isolation of certain lignans (6,7) including cleistanthin A, cleistanthin B, collinusin and diphyllin (Fig.1). The presence of these compounds in the leaves has already been established. Reports on the medicinal utility of the above said compounds have been well documented in our earlier reports (8-11).

The four lignans cited earlier, due to their high aromaticity, exhibit brilliant luminescence when irradiated under U.V. and serve as excellent model compounds to be followed fluorometrically. Based on their photometric properties (including fluorescence) photometric (UV and visible), fluorometric, solid state fluorodensitometric and photodensitometric methods were developed in our laboratory (8-11). The spectral characteristics are presented in Table 1. The present communication deals

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CHEMICAL STRUCTURES AND R_F VALUES OF LIGNAN LACTONES FROM THE LEAVES OF C.COLLINUS

COMPOUND	STRUCTURE	R_F VALUE*
CLEISTANTHIN A	R = 3, 4 di-O- methyl xylose; OR; MeO; MeO; O; O; O; O	0·37
CLEISTANTHIN B	OR; MeO; MeO; O; O; O; O; R = glucose	0·01
DIPHYLLIN	OH; MeO; MeO; O; O; O; O	0·27
COLLINUSIN	MeO; MeO; O; O; O; O	0·55

*Tlc on kieselgel 60 G with n-Heptane-Chloroform Ethanol (50 : 50 : 5) as mobile system.

FIGURE 1

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TABLE 1

SPECTRAL CHARACTERISTICS OF C.COLLINUS LIGNANS

	UV λ_{max}	Visible* λ_{max}	Fluorometry λ_{Excit}	λ_{Emis}
	nm			
Cleistanthin A	262	580	356	441
Cleistanthin B	262	580	322	440
Diphyllin	268	580	362	432
Collinusin	250	580	**	**

* Coloured complex with chromotropic acid

** No response upto 600 μg/3ml

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with the high-pressure liquid chromatographic separation of the four lignans, the "active principles" of the poisonous plant C.collinus.

EXPERIMENTAL

Instrument:

A Pye Unicam (Philips, Cambridge, UK) Liquid Chromatograph (PU 4800) equipped with video chromatographic control centre was used.

Standard Solutions:

Authentic samples of cleistanthin A, collinusin and diphyllin were obtained from M/S Ciba (Bombay) and cleistanthin B from Osmania University (Hyderabad). The purity of the samples were checked from their spectral and chromatographic data.

Standard solutions of the four lignans were prepared in ethanol (500 µg/ml) and diluted suitably as and when needed.

Solvents were of analar/spectral grade quality and used after degassing. Water used in the mobile phase is all-glass double distilled water.

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Chromatographic Conditions:

Column: Spherisorb-5 ODS (25 mm x 4.6 mm i.d)

Mobile phase: Methanol, Water (73-27) isocratic system

Flow rate: 1 ml min^{-1}

Oven temperature: 34°C

Injector: 20 µl capillary loop

Detection: a) UV detector - Pye Unicam variable wavelength (PU 4020) set at 262 nm

b) Fluorescence detector: Fluorichrom (Varian, U.S.A) equipped with excitation filter 340-380 nm and emission filter above 400 nm. The lamp and gain were set at low position and the attenuator at 1 (most sensitive).

HPLC Separation:

Standard samples of the four lignans (0.25 µg) were injected individually under the described chromatographic

conditions and the retention times were recorded. This was repeated to observe the reproducibility in retention time and followed by a mixture of all the four (0.25 µg each). The above procedures were repeated for both HPLC-fluorometry and HPLC-UV methods.

Preparation of Leaf Extract:

C.collinus leaf extract was prepared from dried leaves (1 g) as described earlier (9) and the final residues taken in an aliquot of ethanol and subjected to HPLC.

Recovery from Spiked Biospecimens:

Blood samples (Rabbit) were spiked with a known quantity of the mixture of four lignans and processed as reported (9). An aliquot of the final extract in ethanol was subjected to HPLC separation.

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RESULTS

The HPLC chromatograms of the mixture of four lignans as shown by the HPLC-fluorometry and HPLC-UV detectors are shown in Fig. 2 and 3. The retention time and the capacity ratio (k') value of the four compounds are given in Table 2.

Calibration Curve

In order to establish the linearity of the methods developed, calibration graphs were constructed for all the four compounds individually as the sensitivity differs for the four lignans. The linearity extends in the range of 0.05 to 0.4 µg for diphyllin, 0.1 to 0.7 µg

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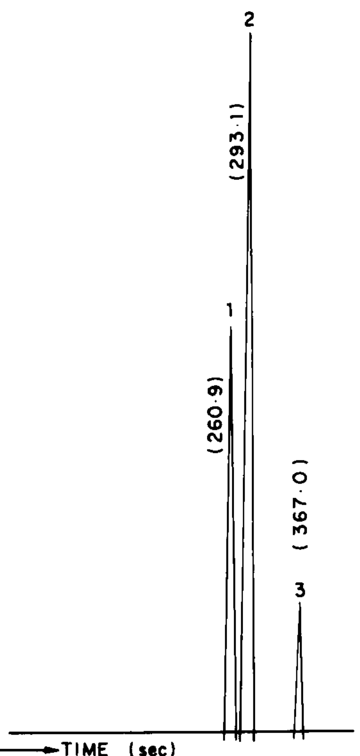

HPLC-FLUORESCENT CHROMATOGRAM OF MIXTURE OF AUTHENTIC SAMPLES (0·25 μg each) ON REVERSEDPHASE COLUMN (SPHERISORB-5 ODS). MOBILE PHASE :- METHANOL : WATER (73 : 27)

PEAK IDENTIFICATION :- 1 CLEISTANTHIN B
2 DIPHYLLIN
3 CLEISTANTHIN A

FIGURE 2

for cleistanthin B and 0.4 to 1.2 μg for cleistanthin A in HPLC-fluorometry. In the case of HPLC-UV the range is .05 to 0.5 μg for cleistanthin B, 0.1 to 0.7 μg for cleistanthin A and 0.2 to 1.2 μg for collinusin and diphyllin.

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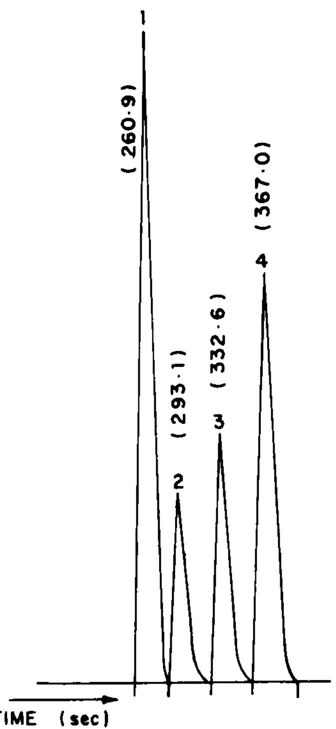

HPLC - ULTRAVIOLET CHROMATOGRAM OF MIXTURE OF AUTHENTIC SAMPLES (0·25 µg each) ON REVERSEDPHASE COLUMN (SPHERISORB - 5 ODS). MOBILE PHASE :- METHANOL : WATER (73 : 27)

PEAK IDENTIFICATION :- 1 CLEISTANTHIN B
2 DIPHYLLIN
3 COLLINUSIN
4 CLEISTANTHIN A

FIGURE 3

TABLE 2

RETENTION TIME AND CAPACITY - RATIO (k') VALUES OF C.COLLINUS LIGNANS ON SPHERISORB - 5 ODS COLUMN

Compound	Retention Time (sec)	k'
Cleistanthin B	260.9	0.26
Diphyllin	293.1	0.42
Collinusin	332.6	0.61
Cleistanthin A	367.0	0.78

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Sensitivity

The limit of detection of these compounds in both the methods, along with a comparison of sensitivity obtained for different techniques, is provided in Table 3.

Reproducibility

The precision of the method was verified by carrying out the experiments repeatedly over a period of time and the reproducibility is expressed as % C.V. in Table 4.

HPLC of Leaf Extract:

The HPLC-fluorometry and chromatograms of the leaf extract of C.collinus are shown in Fig. 4 and 5. The peaks corresponding to the four lignans are marked. As evident, the leaf extract contains a few more fluorescing and UV absorbing compounds, yet to be identified. A thin-layer

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TABLE 3

COMPARISON OF SENSITIVITY IN DIFFERENT TECHNIQUES

Compound	Ultraviolet	Spectro Fluorometry	Fluoro-Densitometry	HPLC Fluorometry	HPLC Ultraviolet
	(MICROGRAM)				
Cleistanthin A	1	1	0.25	0.4	0.1
Cleistanthin B	1	1	0.1	0.1	0.05
Diphyllin	1	0.1	0.25	0.05	0.2
Collinusin	1	*	0.25	**	0.2

* No response upto 600 µg/3ml

** No response upto 10 µg

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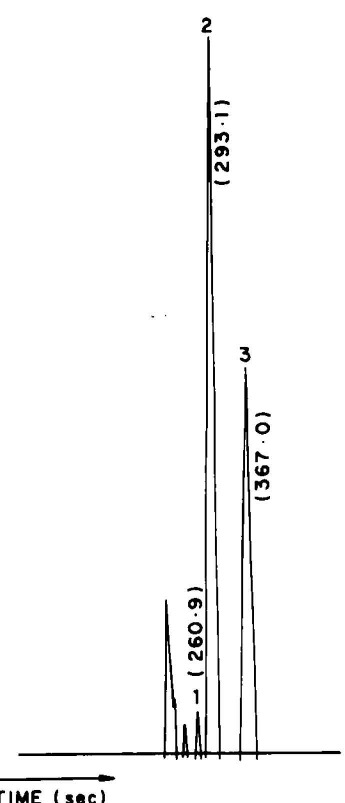

HPLC-FLUORESCENT CHROMATOGRAM OF C. COLLINUS LEAF EXTRACT ON REVERSED PHASE COLUMN (SPHERISORB-5 ODS). MOBILE PHASE :- METHANOL : WATER (73 : 27).

PEAK IDENTIFICATION : 1 CLEISTANTHIN B
2 DIPHYLLIN + UNKNOWN COMPOUND
3 CLEISTANTHIN A

FIGURE 4

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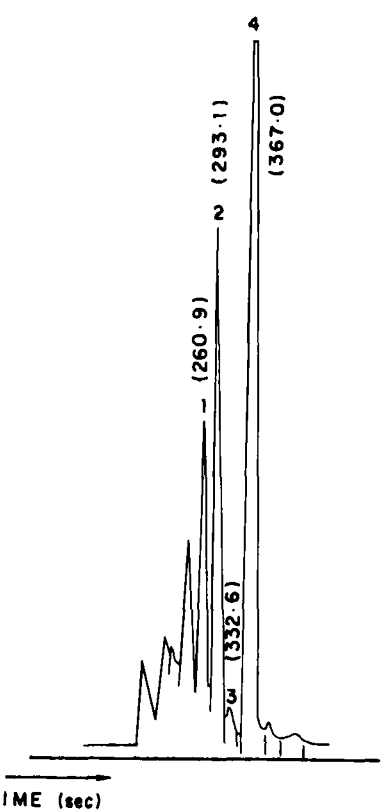

HPLC-ULTRAVIOLET CHROMATOGRAM OF C. COLLINUS LEAF EXTRACT ON REVERSEDPHASE COLUMN (SPHERISORB - 5 ODS). MOBILE PHASE :- METHANOL : WATER (73 : 27).

PEAK IDENTIFICATION :- 1 CLEISTANTHIN B
2 DIPHYLLIN + UNKNOWN COMPOUND
3 COLLINUSIN
4 CLEISTANTHIN A

FIGURE 5

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TABLE 4

REPRODUCIBILITY OF THE QUANTITATIVE ANALYSIS OF C.COLLINUS LIGNANS (EACH 0.50 μg) BY REVERSED PHASE HPLC

Compound	Standard Deviation		Co-efficient Variation	
	Within the Day	Day-to-Day	Within the Day	Day-to-Day
Cleistanthin A	0.34	0.48	1.16	1.64
Cleistanthin B	0.26	0.77	0.60	1.82
Collinusin	0.24	1.43	1.88	9.70
Diphyllin	0.31	0.64	2.28	5.02

chromatogram of the leaf extract along with the four lignans on kieselgel 60 G is shown in Fig. 6. The eluates from HPLC-column corresponding to the four lignans were monitored by TLC and photometry to check their purity.

Recovery from Spiked Biological Samples:

The percentage recovery of the lignans from spiked blood samples is shown in Table 5.

DISCUSSION

As shown in Fig. 2 and 3, the four compounds were well separated under the conditions described. Different binary and ternary solvent systems such as acetonitrile-water, ethanol - water, chloroform - methanol, acetonitrile - methanol - water were tried as mobile phase. But methanol - water offered the best resolution. Different ratio of water in the mobile phase, from 5 to 40% were tried and 27% was found to give the optimum separation, both from the view of sensitivity and resolution. Increasing the water content in the mobile phase decreased the sensitivity, especially in HPLC-fluorometry.

However, no definite explanation could be offered for the order of resolution as there is no correlation between molecular structure and retention time. As explained by Kim and Ayres (1) in the separation of aryletrahydronaphthalene, the relative order of affinity towards hydrophobic centres could be one of the contributing factors in deciding the order of elution from the column.

As seen in Fig. 2 only cleistanthins A, B and diphyllin alone were detected but not collinusin in HPLC-

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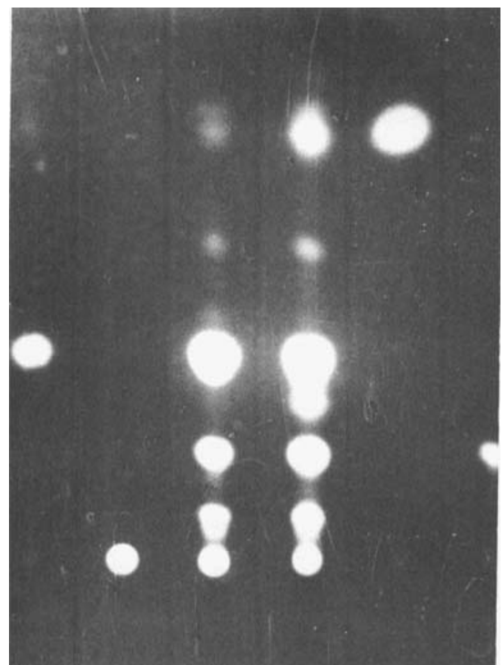

FIGURE 6

UV PHOTOGRAPH OF THIN LAYER CHROMATOGRAPHIC RESOLUTION OF C.COLLINUS LIGNAN LACTONES

From L to R

1. Cleistanthin A
2. Cleistanthin B

3&4. Leaf extract of C.collinus*

5. Collinusin
6. Diphyllin

*C.collinus leaves were collected from the different places separated by a distance of 200 km.

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TABLE 5

RECOVERY OF C.COLLINUS LIGNANS FROM SPIKED BLOOD SAMPLE BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY - FLUOROMETRY*

Specimen	Diphyllin		Cleistanthin B		Cleistanthin A	
	Amount Added (μg)	% Recovery	Amount Added (μg)	% Recovery	Amount Added (μg)	% Recovery
Blood	5	94.4	5	93.7	5	93.1

* Each value is average from three determinations.

fluorometry. This was the case up to a concentration of 10 µg. This observation is corroborative with our earlier one in spectrofluorometry where there was no response up to a concentration of 600 µg/3 ml as given in Table 3. As in spectrofluorometry, diphyllin offerd the maximum sensitivity in HPLC-fluorometry followed by cleistanthin B and cleistanthin A. It is known that the intensity of fluorescence and sensitivity of the four lignans differ in solution and solid state (on silica gel) (10). Collinusin, however, could be detected at as low as 0.25 µg level with the intensity of fluorescence remaining unaltered for several days fluorodensitometrically. In the case of HPLC-UV, all the four lignans could be monitored at 262 nm simultaneously, cleistanthin B offering the maximum sensitivity followed by cleistanthin A, collinusin and diphyllin. Of the two methods HPLC-fluorometry is the preferred one due to the absence of interference from endogenous compounds (Fig. 7).

Analysis of the fractions collected from the column effluent by thin-layer chromatography (10) revealed that diphyllin could not be estimated in the leaf extract (Fig. 4 and 5) due to the interference of another fluorescing compound whose structure has not been established. Attempts are being under way to overcome this difficulty by using a gradient system and adsorption columns. But this is still not a serious problem in clinical and forensic toxicology becuase cleistanthin A

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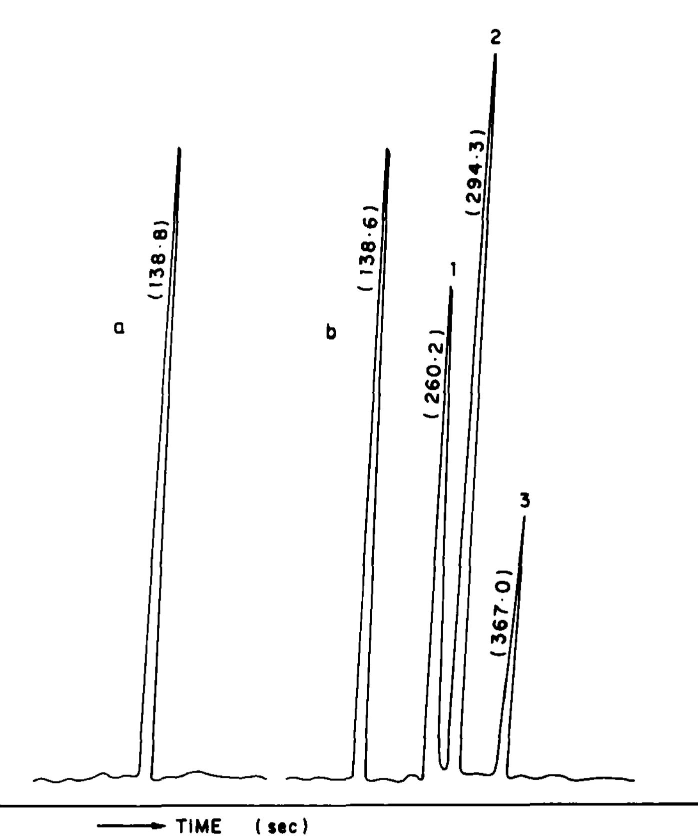

HPLC - FLUORESCENT CHROMATOGRAM OF
a) RABBIT BLOOD (BLANK, 20 µl EXTRACT)
b) RABBIT BLOOD (20 µl SPIKED WITH MIXTURE OF C. COLLINUS LIGNANS 0·50 µg EACH)
COLUMN :- SPHERISORB - 5 ODS
MOBILE PHASE :- METHANOL : WATER (73 : 27)
PEAK IDENTIFICATION :- 1 CLEISTANTHIN B
2 DIPHYLLIN
3 CLEISTANTHIN A

FIGURE 7

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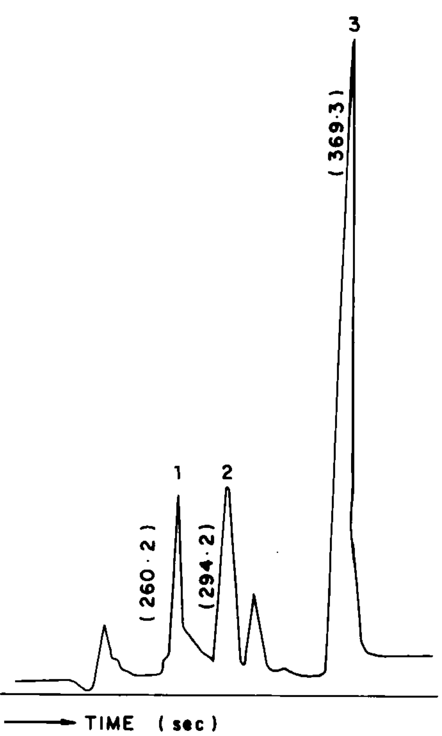

HPLC-FLUORESCENT CHROMATOGRAM OF "ODUVIN" ISOLATED FROM THE LEAVES OF C.COLLINUS.

COLUMN :- SPHERISORB - 5 ODS
MOBILE PHASE :- METHANOL : WATER (73 : 27)
PEAK IDENTIFICATION :- 1 CLEISTANTHIN B
2 DIPHYLLIN
3 CLEISTANTHIN A

FIGURE 8

is the major constituent of C.collinus leaf and also reported to be highly toxic (6). Extensive investigation with the crude poisonous principles isolated earlier, such as "oduvin" (12), "principle A", "principle B" (13) etc., showed the presence of cleistanthin A to be the major constituent. The HPLC- fluorometry chromatogram of 'oduvin' is shown in Fig.8. The importance of quantifying cleistanthin A is again emphasized from the fact that the bio-distribution studies of C.collinus lignans in animals revealed the presence of cleistanthin A even after 24 h. This fraction collected from the column effluent was tested for its purity by TLC with different systems and by spectral data.

The present HPLC method can thus be readily adopted in clinical and forensic toxicology. For chemotaxonomical and pharmacological studies fluorodensitometric method is suggested.

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