

Evaluation of Purity of some Coumarin Derivatives by Melting Point Measurements, TLC and Scanning Densitometry

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Abstract: Impurity remains after syntheses greatly influence properties and effectiveness of synthesized compounds. Therefore it is very important to determine its presence and remove them from the synthesized product. In order to obtain crystals of high purity, synthesized bis-4-hydroxycoumarins and benzopyranocoumarins were purified by recrystallization and column chromatography. The success of the purification of the synthesized compounds was examined by the melting point measurements, thin layer chromatography and scanning densitometry. Only one of nine tested mobile phase systems proved successful for separation of the analyzed compounds. Detection of the compounds on TLC plates was carried out under UV light at wavelength of 254 nm, as well as quantitative analysis of the present impurities. The results of scanning densitometry showed that the corresponding compounds obtained by purification had a satisfactory degree of purity (less than 2% unknown impurities). Percentage of impurities can also be calculated from data attained by spectrometric methods and elemental analysis.

INTRODUCTION

It is very important to identify and quantify impurities in products. This represents a crucial step in quality control of new drugs, as much as, active compounds. Different mechanisms of degradation of drugs can lead to a very wide spectra of possible effects such as: loss of pharmaceutical quality (loss or increase of an active compound's concentration, change in uniformity of content, endangering of microbiological status, forming toxic degradation products), changes in bioavailability and also a modification of any other factor that can represent a threat to the validity of drug.

(Jiben, 2002; Vijayalakshmi, Kumaravel and Anbazhagan, 2012).

Impurities in an active compound can occur during the synthesis, purification and during the validity time. They can be classified as: inorganic impurities (reagents, catalysts, ligands, inorganic salts, heavy metals or other residual metals), organic impurities (starting materials, process-related impurities, intermediates, and degradation products) and residual solvents (organic and inorganic liquids). (ICH. Q3A (R2), 2006; Lunn and Faustino, 2007).

Impurities that remain after the synthesis greatly affect the properties and efficiency of mentioned synthesized compound. In order to get crystals with a high degree of purity, synthesized compounds must undergo purification with different procedures such as recrystallization and chromatography methods (column chromatography, HPLC, TLC). (Görög, 2000 a).

There are two major approaches to determine the purity of a chemical compound. (Görög, 2000b; Beckett and Stenlake, 1988). The first is the absolute approach, based on thermodynamic methods; the total amount of impurities can be determined without detailed knowledge of these impurities. The second is the chromatographic approach (TLC, HPLC, GC), which gives information about the detection and determination of impurities as far as their nature and chemical behavior are known. (Ferenczi-Fodor, Vegh and Renger, 2006; Nyiredy, 2001; Nageswara Rao and Nagaraju, 2003). Chromatographic methods can be applied for the determination of purity due to their many advantages such as high efficiency, speed and the possibility of their utilization in automatized systems. Investigation and quantification of impurities presented in synthesized coumarin derivatives were achieved by measuring melting points, and using thin layer chromatography and scanning densitometry. (Gumieniczek, Inglot and Kończak, 2011).

EXPERIMENTAL

General procedures for the preparation of compounds

Benzylidene-bis-(4-hydroxycoumarin) derivatives (**1-2**) and fused benzopyranocoumarin derivatives (**3-4**) were prepared in moderately good yields by Claisen condensation of 4-hydroxycoumarin (4-HC in further text) (Sigma Aldrich, 98%) and appropriate substituted aromatic aldehydes, all being commercially available [4-bromobenzaldehyde (Merck, 98%), 4-(trifluoromethyl) benzaldehyde (Sigma Aldrich, 98%), 2-fluoro benzaldehyde (Sigma Aldrich, 97%) and 5-bromo-2-hydroxybenzaldehyde (Merck, 97%)].

According to the previously published procedure (Završnik, Muratović, Makuc, *et al.*, 2011) 4-HC (0.324 g, 2 mmol) was dissolved in hot ethanol (6 mL) (Sigma Aldrich, puriss. p.a., absolute, $\geq 99.8\%$), the appropriate aldehyde (1 mmol) was added, and the reaction mixture was refluxed for 24 h. After cooling to room temperature, the solid was filtered out and purified in parallel by recrystallization and column chromatography to give products benzylidene-bis-(4-hydroxycoumarin) derivatives **1** and **2**, and 3-(6-oxo-(1*H*)-benzopyrano[4,3-*b*]benzopyran-7-yl)-4-hydroxycoumarin derivatives **3** and **4** (Figure 1).

General procedures for analytical and experimental section

Melting points (uncorrected) were determined with Büchi melting point B-545. Precoated silica gel 60F-254 plates were used for thin-layer chromatography (TLC); samples were applied to plate by CAMAG Linomat 5

and the spots were detected and scanned by CAMAG TLC Scanner 3, under UV light of wavelength 254 nm. Column chromatography was performed using silica gel 60 (0.063-0.2 mm); glass column was slurry-packed under gravity. Solvent system used for TLC and column chromatography was dichloromethane (Sigma Aldrich, puriss. p.a., 99.6%) / ethyl acetate (Sigma Aldrich, puriss. p.a., 99.6%) = 2:1. Solvent used for recrystallization was chloroform (Sigma Aldrich, puriss. p.a., 99.0-99.4%).

Mass spectra were recorded with an Autospec: ESI/Q-TOF Premier instrument. ^1H -NMR spectra were recorded at 600 MHz, in deuterated DMSO-*d*₆ (Sigma Aldrich, 99.8%), on Bruker (UXNMR/XwinNMR) spectrometer, using tetramethylsilane (TMS) as internal reference. 1D and 2D NMR spectra were recorded at 25 °C on Varian Unity Inova 300 spectrometers. ^1H chemical shifts were referred to residual signal of CDCl₃, CD₂Cl₂ and DMSO-*d*₆ (δ TMS 0.0 ppm). Elemental analyses were performed in the Central Analytic Service, Ruđer Bošković Institute Zagreb, Croatia, using a Perkin Elmer 2400 Elemental Analyser. The infrared spectra were obtained from potassium bromide triturate containing 0.5% of the product on a Perkin-Elmer 1000 FT-IR spectrophotometer. All data were recorded at 25 °C unless specified otherwise.

Determination of purity with TLC

Thin-Layer Chromatography (Nyiredy, 2001) is a simple and inexpensive technique that is often used to judge the purity of a synthesized compound or to indicate the extent of progress of a chemical reaction. The analysis was performed on precoated 20x20 cm (0.25 mm thick) 60F-254 silica gel TLC plate. Nine different mobile phases (Table 1.) were selected according to their polarity to establish the R_f value for investigated compounds.

Table 1. Mobile phase systems studied

	Solvent system	v/v
1	toluene : chloroform : acetone	8 : 8 : 1
2	hexane : acetone : glacial acetic acid	5 : 5 : 2
3	cyclohexane : chloroform : glacial acet. acid	5 : 5 : 2
4	cyclohexane : chloroform : glacial acet. acid	15 : 15 : 4
5	n-hexane : acetone	2 : 1
6	dichloromethane : ethyl acetate	2 : 1
7	n-hexane : ethyl acetate	11 : 3
8	dichloromethane : methanol	9 : 1
9	toluene : chloroform : acetone	8 : 8 : 1

On TLC plate 10 μL of probe solutions were applied as spots 6 mm length and 0,45 mm width band. The plates were than dried at room temperature and afterwards were developed in a vertical chamber previously saturated with mobile phase vapors for 60 minutes, at room temperature. The development distance was 4/5 of

a plate. After development, the plates were dried on hot plate. The spots were detected and scanned under UV light (wavelength 254 nm) automatically by CAMAG TLC Scanner 3. The R_f value is the identification characteristic in TLC and depends on the used combination of solvents and the chemical nature of substance.

RESULTS AND DISCUSSION

Chemistry

Synthesis and characterization of four biscoumarin compounds were described in previously published paper (Završnik, Muratović, Makuc, *et al.*, 2011). Chemical structure of bis-4-hydroxycoumarins and benzopyrano- coumarins are presented on Figure 1.

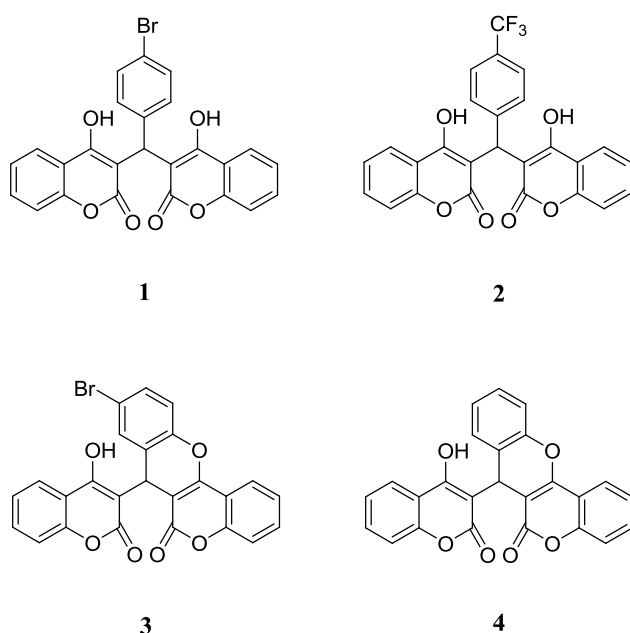


Figure 1. Benzylidene-bis-(4-hydroxycoumarin) derivatives (**1** and **2**) and 3-(6-oxo-(1H)-benzopyrano[4,3-b]benzopyran-7-yl)-4-hydroxy-coumarin derivatives (**3** and **4**)

3,3'-(4-Bromobenzylidene)-bis-(4-hydroxycoumarin)

(**1**) (Završnik, Muratović, Makuc, *et al.*, 2011).

Yield= 74%. mp 267.2 °C. Anal. calcd for $C_{25}H_{15}O_6Br$: C 61.12, H 3.08. Found: C 61.07, H 3.13. All values are given in percentages. IR (KBr) ν 3276 2730 1772 1668 1604 1564 1488 1454 646 cm^{-1} . 1H -NMR (600 MHz, DMSO- d_6): δ 6.02 (s, 1H, H*), 7.10 (m, 2H, H-2' and H6'), 7.38 (m, 2H, H-6 and H-6''), 7.41 (m, 2H, H-8 and H8''), 7.44 (m, 2H, H-3' and H-5'), 7.64 (m, 2H, H-7 and H-7''), 7.99 (d, J 7.7, 1H, H-5 and H-5''), 8.07 (d, J 7.3, 1H, H-5 and H-5''), 11.32 (s, 1H, OH), 11.54 (s, 1H, OH). MS m/z : 491 (M^+H , 100).

3,3'-(4-Trifluoromethylbenzylidene)-bis-(4-hydroxycoumarin) (**2**) (Završnik, Muratović, Makuc, *et al.*, 2011).

Yield 72%. Mp 271.3 °C. Anal. Calcd for $C_{26}H_{15}O_6F_3$: C 65.01, H 3.15, F 11.86. Found: C 65.11, H 3.25, F 11.97. All values are given in percentages. IR (KBr) ν 2918 1778 1652 1470 1198 1028 cm^{-1} . 1H -NMR (600 MHz, DMSO- d_6): δ 6.34 (d, J 0.9, 1H, H*), 7.27 (m, 2H, H-6 and H-6''), 7.31 (m, 2H, H-8 and H-8''), 7.34 (d, J 8.4,

2H, H-2' and H-6'), 7.55 (m, 2H, H-7 and H-7''), 7.55 (d, J 8.4, 2H, H-3' and H-5'), 7.85 (dd, J 7.8, 1.5, 2H, H-5 and H-5''). ^{19}F -NMR (DMSO- d_6): δ -60.03 (s, CF_3). MS m/z : 481 (M^+H , 100).

3-(6-Oxo-(1H)-18-bromobenzopyrano[4,3-b]benzopyran-7-yl)-4-hydroxycoumarin (**3**) (Završnik, Muratović, Makuc, *et al.*, 2011).

Yield 72%. Mp 296 °C. Anal. Calcd for $C_{25}H_{13}O_6Br$: C 61.9, H 2.68. Found: C 61.7, H 2.68. All values are given in percentages. IR (KBr) ν 3274 2886 1772 1610 1570 1558 1496 1220 586 cm^{-1} . 1H -NMR (600 MHz, DMSO- d_6): δ 5.71 (s, 1H, H*), 7.31 (d, J 1.8, 1H, H-6'), 7.32 (m, 1H, H-8), 7.34 (d, J 8.7, 1H, H-3'), 7.36 (m, 1H, H-6), 7.45 (m, 1H, H-8''), 7.49 (m, 1H, H-6''), 7.52 (m, 1H, H-4'), 7.61 (m, 1H, H-7), 7.71 (m, 1H, H-7''), 8.03 (b, 1H, H-5), 8.11 (dd, J 7.9, 1.4, 1H, H-5''), 12.19 (b, 1H, OH). MS m/z : 488 (M^+H , 100).

3-(6-Oxo-(1H)-benzopyrano[4,3-b]-(1)10-hydroxybenzo-pyran-7-yl)-4-hydroxycoumarin (**4**) (Završnik, Muratović, Makuc, *et al.*, 2011).

Yield 82%. Mp 241.7 °C. Anal. Calcd for $C_{25}H_{14}O_6$: C 73.17, H 3.43. Found: C 72.7, H 2.99. All values are given in percentages. IR (KBr) ν 2958 1616 1570 1488 1456 1230 cm^{-1} . 1H -NMR (600 MHz, DMSO- d_6): δ 5.74 (s, 1H, H*), 7.14 (m, 1H, H-5'), 7.20 (m, 1H, H-6'), 7.32 (m, 1H, H-8), 7.34 (m, 1H, H-4'), 7.35 (m, 1H, H-3'), 7.36 (m, 1H, H-6), 7.46 (m, 1H, H-8''), 7.49 (m, 1H, H-6''), 7.60 (m, 1H, H-7), 7.71 (dd, J 7.5, 1.7, 1H, H-7''), 8.03 (m, 1H, H-5), 8.11 (dd, J 7.9, 1.4, 1H, H-5''), 12.21 (b, 1H, OH). MS m/z : 411 (M^+H , 100).

TLC and scanning densitometry

Analyzed compounds were chromatographed with each of nine mobile phases that were chosen according to their polarity. Only a mobile phase **6** of those tested (Table 1.), yielded successful separation of synthesized compounds, whilst other mobile phases were less efficient. Results are presented for the mobile phase **6** (dichloromethane/ethyl acetate = 2:1) and are shown on Figure 2. Plate was investigated under the UV light at wavelength of 254 nm.



Figure 2. TLC plate developed with mobile phase **6**, under the UV light on 254 nm for the compounds **1** – **4** (raw substances (**A**) and after purification process (**B**); 4-HC at the end)

Thin layer chromatography can be applied for the quantitative analysis of samples and possible impurities. TLC plates were analyzed with scanning densitometer, in order to get quantitative data on percentage of present impurities. Resulting densitogram is presented on Figure 3.

Use of this technique allows identification of analyzed compounds not only by usual comparison of R_f values with standard compounds but also by comparison of their absorption or fluorescence spectra. Quantification is based on comparison of height or area under chromatographic peak (curve) of analyzed sample with height or area under the peak of standard on the same plate. Quantitative analyses of each compound from recorded densitogram are presented on Table 2.

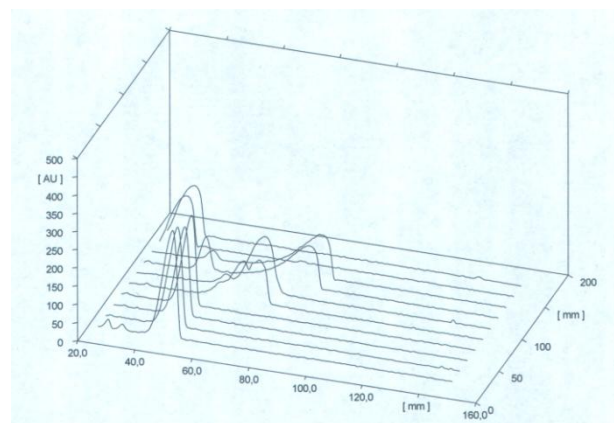


Figure 3. Densitogram recorded after TLC plate scanning at 254 nm in the absorbance mode

Table 2. Quantitative analysis after densitometry scanning for synthesized compounds and 4-HC

Peak	Start mm	Start Height	Max mm	Max Height	Max %	End mm	End Height	Area	Area %	Assigned substance
RAW COMPOUND 1 (1A)										
1	28.6	0.3	31.6	16.5	4.9	34.0	0.7	379.5	2.1	4-HC
2	39.3	3.7	49.3	294.5	88.1	52.6	1.9	16966.1	95.2	1
PURIFIED COMPOUND 1 (1B)										
1	24.2	1.9	24.9	3.9	1.4	26.2	1.0	54.1	0.4	4-HC
2	37.9	3.5	48.3	273.7	96.8	52.2	2.1	15406.7	99.2	1
RAW COMPOUND 2 (2A)										
1	23.5	0.7	25.2	5.6	2.2	28.1	0.2	125.9	1.0	4-HC
2	38.1	1.1	47.9	248.1	97.8	51.7	1.4	12942.2	99.0	2
PURIFIED COMPOUND 2 (2B)										
1	24.9	1.0	27.0	4.0	1.6	28.4	0.3	74.3	0.6	4-HC
2	38.9	3.4	48.2	245.2	98.4	52.4	1.7	12986.4	99.4	2
RAW COMPOUND 3 (3A)										
1	24.2	1.3	29.6	8.2	3.3	32.2	1.8	270.0	1.6	4-HC
2	49.3	9.0	63.6	111.4	45.4	65.2	89.8	9283.1	54.4	unknown
3	65.3	90.0	68.9	120.8	49.3	73.7	4.5	7350.3	43.1	3
PURIFIED COMPOUND 3 (3B)										
1	26.0	0.9	30.0	4.6	2.2	31.0	2.7	147.7	0.8	4-HC
2	48.8	7.5	54.6	35.2	16.9	57.0	25.0	1873.6	10.3	unknown
3	57.1	25.1	68.2	155.1	74.3	76.3	3.7	15921.2	87.6	3
RAW COMPOUND 4 (4A)										
1	23.3	0.4	24.7	5.5	2.4	26.0	0.1	246.4	1.2	4-HC
2	37.6	2.3	44.8	95.8	42.0	48.1	10.3	4197.5	20.5	unknown
3	61.0	18.1	80.9	115.9	50.9	85.9	2.0	15905.2	77.8	4
PURIFIED COMPOUND 4 (4B)										
1	38.2	1.2	44.7	29.3	19.6	48.1	6.6	1354.6	7.6	unknown
2	64.5	23.9	82.2	119.9	80.4	87.8	0.3	16440.2	92.4	4
4-HC										
1	23.3	1.4	32.4	136.5	92.4	36.5	0.2	11309.0	97.3	4-HC
2	70.1	0.3	72.8	6.4	4.3	75.5	1.5	196.0	1.7	unknown

As seen from Table 2. purification process by column chromatography was successful for these raw synthesized compounds. Analysis unambiguously showed increase in main compound percentage after purification process and decrease in percentage of 4-HC and other unknown impurities (present in some of the samples), indicating higher purity (bold column "Area %" in Table 2.). Impurities likely come from 4-HC itself as a reagent (having in mind its labeled purity percentage and results from Table 2.) or represent byproducts of syntheses.

CONCLUSIONS

Investigated compounds were analyzed on TLC plates with nine different mobile phases. Only mobile phase dichloromethane/ethyl acetate = 2:1 showed good separation of analyzed compounds. Plates were detected under the UV light at wavelength of 254 nm. Scanning densitometry analysis of present impurities was performed by scanning of TLC plate at 254 nm as well. Results of scanning densitometry showed that purification processes yielded compounds of higher purity.

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Summary/Sažetak

Onečišćenja koja zaostaju nakon sinteza u velikoj mjeri utiču na svojstva i djelotvornost sintetiziranog spoja, te je od velikog značaja utvrđivanje njihovog prisustva i uklanjanje iz sintetiziranog produkta. U cilju dobivanja kristala visokog stepena čistoće, sintetizirani spojevi bis-4-hidroksikumarina i benzopiranokumarina prečišćeni su postupcima prekrystalizacije i kolonske hromatografije. Uspješnost prečišćavanja sintetiziranih spojeva ispitana je određivanjem tački topljenja, te hromatografijom na tankom sloju i skenirajućom denzitometrijom. Samo jedna od devet ispitivanih kombinacija mobilnih faza se pokazala dovoljno uspješnom za razdvajanje ispitivanih spojeva. Detekcija ploča je provedena pod UV svjetlom na talasnoj dužini 254 nm, kao i denzitometrijska kvantitativna analiza prisutnih onečišćenja. Rezultati skenirajuće denzitometrije su pokazali da su odgovarajućim prečišćavanjem dobiveni spojevi zadovoljavajućeg stepena čistoće (manje od 2% nepoznatih onečišćenja). Procenat čistoće se također može izračunati i iz podataka dobivenih spektroskopskim metodama i elementarnom analizom.