Determination of relative response factors of the opium alkaloids with HPLC-DAD

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Abstract

In this work, a convenient method for determination of relative UV response factors (RRFs) of morphine, codeine, thebaine, oripavine, noscapine and papaverine by high performance liquid chromatography (HPLC) equipped with a diode array detector (DAD) was presented. Pholcodine was selected as the reference compound for calculating the relative response factors of the alkaloids.

The separation of all seven compounds was obtained with optimized gradient elution with high pH value of the mobile phase on a reversed phase column with bidentate C18-C18 bonding technology. The RRFs of the alkaloids were determinate by three different approaches: 'regression analysis/mass concentration', 'regression analysis/molar concentration'and 'detector sensitivity' approaches.

The 'regression analysis/molar concentration' approach gave the accurate approximation of the exact amount of the substance that enters in the detector and the statistically relevant calculation includes several points of different concentrations (at least five), which makes this approach most advantageous one.

This method is suitable for quality assessment of the standardised opium dry extract, raw opium and standardised opium tincture by quantitative analysis of not only morphine and codeine as indicated in the respective European Pharmacopoeia monographs, but as well as the major impurities that originate from opium poppy *Papaver somniferum* L. (Papaveraceae).

Key words: HPLC-DAD, Relative response factors (RRFs), opium alkaloids, internal standard

Introduction

The European Pharmacopoeia, in the monographs for the Opium dry extract, standardised (01/2008:1839); Opium, raw (01/2008:0777); and Opium tincture, standardised (01/2008:1841) provides HPLC-UV assay method for morphine and codeine and test method for thebaine (Ph.Eur.7). These natural products, however, can contain even more alkaloids that originate from opium poppy *Papaver somniferum* L. (Papaveraceae), including: noscapine papaver-ine and oripavine (Fig. 1). These alkaloids are expected to be identified with the TLC method provided for identification in the cited monographs.

All of the above mentioned alkaloids are important for the quality of the cited natural products described in the monographs. The presence of some alkaloids other than those defined in the content can be considered as impurities if their pharmacological action is different from the desired one.

Providing reference standard for all the analytes of interest in natural products is sometimes difficult, especially for the non pharmacopoeial substances, or substances which are subject to international narcotics control, so the content determinations often include quantitation based

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on relative HPLC peak areas obtained at a specific wavelength. In order for this quantitation to accurately reflect weight percentages of the compounds, the relative UV response factors (absorptivities) at the given wavelength must be known (Sun et al., 2008; Ph.Eur.2.2.46).

The objective of this study was to determine UV relative response factors (RRf) of opium alkaloids at different wavelengths and to propose a method for rapid, routine analysis for the content determination of six alkaloids in standardised opium dry extract, raw opium and standardised opium tincture.

Experimental

Materials: Authentic samples of morphine, codeine, oripavine, thebaine, noscapine, papaverine and pholcodine (free base each) were obtained from Alkaloid A.D., Skopje (Skopje, R.Macedonia).

Reagents: *Methanol* (HPLC grade), *Trifluoroacetic acid* (for synthesis) and *Phosphoric acid 85%* (pro analysis) were purchased from Merck, Damstad, Germany. *Triethylamine* (pro analysis) was purchased from Sigma-Aldrich, Steinheim, Germany. *Water* (highly purified) was obtained with a TKA-LAB Reinstwasser system (Niederelbert, Germany).

Standard Solution of Alkaloids: Standard solutions of alkaloids were prepared from methanolic stock solutions of 2 mg/ml morphine, 1 mg/ml codeine, 1 mg/ml thebaine, 1 mg/ml oripavine, 1 mg/ml papaverina, 2 mg/ml noscapine and 2 mg/ml pholcodine. Standard solution of alkaloids for a HPLC method was prepared in the same manner to obtain a concentration of 200 μg/ml morphine and pholcodine (as internal standard), 100 μg/ml codeine and noscapine and 50 μg/ml thebaine, papaverine and oripavine.

Chromatographic Conditions: The separation of the six alkaloids and the internal standard was performed on Agilent 1200 HPLC/DAD system and a 250 mm x 4.6 mm i.d., 5 μm particle size, Zorbax Extend C-18 column (Agilent Technologies). The eluents were a solution of 0.1% trifluoroacetic acid (TFA) in water, pH adjusted to 9.6 with triethylamine (TEA) (= solvent A) and methanol (= solvent B), both filtered through 0.45 μm Millipore filters. Elution was performed with optimized gradient (Acevska, 2012 a). The flow rate was 1.5 mL/min and the injection volume 20 μl. The column temperature was maintained at 40ºC. The measurements were made with UV detection at wavelength of 280 nm, and the injection volume was 20μl. Pholcodine was selected as the reference compound for calculating the relative response factors of the alkaloids. The RRFs of the alkaloids were determined by linear calibration curves and through absolute response factors of the alkaloids.

Statistical analysis: The regression analysis was performed using Excel Analysis Tool Pack. The regression curves were obtained by plotting mass concentration or molar concentration of the alkaloids vs. their HPLC peak areas. The significance of the difference (d) between the RRf values obtained by different approaches was calculated using equitation $d = (RRf_{ni} - RRf_{nt})/RRf_{nt} \times 100\%$ at level of significance p=0.05.

Results and discussion

According to Ph.Eur. methods, the detection of morphine and codeine is performed at wavelength of 280 nm. Since the alkaloids have different absorptivities at pH 9.6 (Fig. 2), as for the proposed mobile phase (Acevska et al., 2012 a; Acevska 2012 b), the content determination must be based on RRfs for all the compounds of interest.

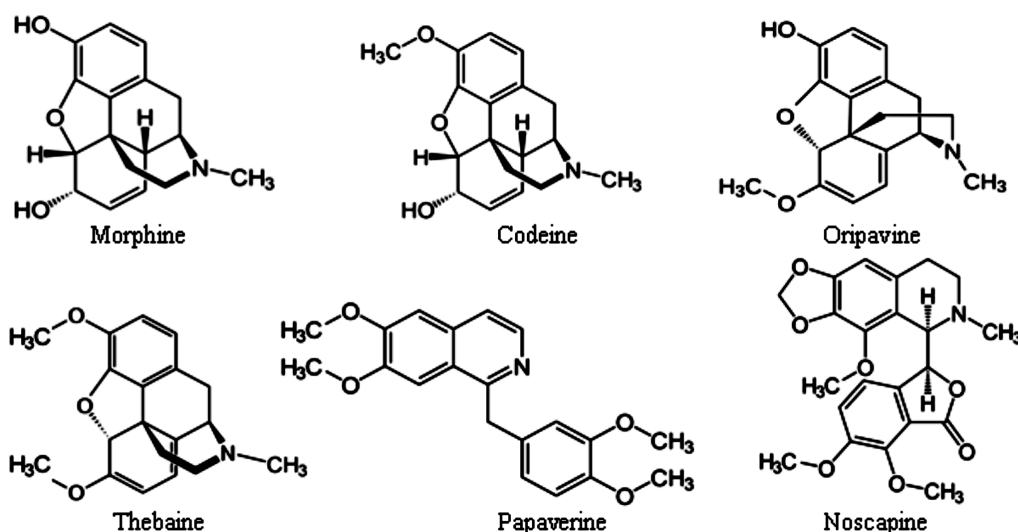The conventional way to determine UV RRf is to an-

Fig.1.   Chemical structure of the alkaloids from opium poppy, *Papaver somniferum* L. (Papaveraceae)
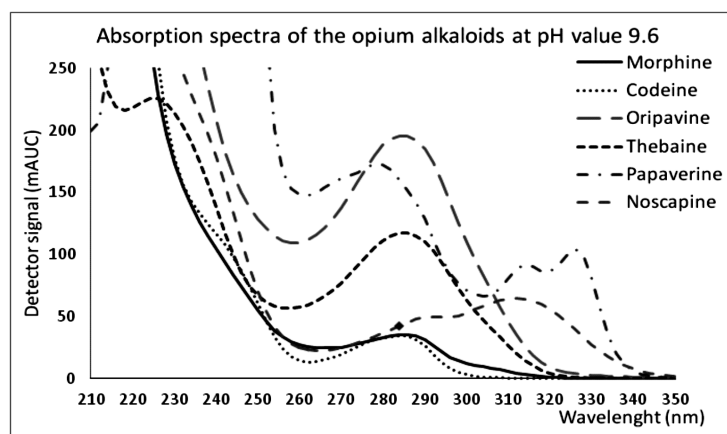
Fig. 2. Absorption maxima of the opium alkaloids at pH value 9.6

Table 1. Regression data (mass concentration) and UV relative response factors of opium alkaloids

Compound	Conc. range (µg/ml)	Regression equitation [a]	RRf[b]	Cf[c]
Morphine	2-300	y=3.56 x+1.69	1.23	0.82
Oripavine	0.5-75	y=18.85x-0.26	6.49	0.15
Codeine	1-150	y=4.00x-1.18	1.38	0.73
Pholcodine	2-300	y = 2.90x + 0.23	1.00	1.00
Papaverine	0.5-75	y=15.14x-2.28	5.21	0.19
Thebaine	0.5-75	y=17.39x-1.37	5.99	0.17
Noscapine	1-150	y=4.92x+0.22	1.70	0.59

[a]The regression curves were obtained by plotting mass concentration (x) vs. peak area (y). [b]RRf is UV relative response factor determined in reference to pholcodine at 280 nm. [c]Cf is correction factor for the quantitation of the alkaloids at 280nm.

Table 2. Regression data (molar concentration) and UV relative response factors of opium alkaloids

Compound	Conc. range (µmol/l)	Molar mass (g/mol)	Regression equitation [a]	RRf[b]	Cf[c]
Morphine	7-1051	285.33	y = 0.0010 x + 1.6873	0.83	1.20
Oripavine	2-252	297.34	y = 0.0056x - 0.2599	4.67	0.21
Codeine	3-501	299.36	y = 0.0012x - 1.184	1.00	1.00
Pholcodine	3-337	398.00	y = 0.0023x + 0.2296	1.00	1.00
Papaverine	1-221	339.29	y = 0.0051x - 2.2833	4.25	0.24
Thebaine	2-241	311.37	y = 0.0054x - 1.3733	4.50	0.22
Noscapine	2-363	413.43	y = 0.0020x + 0.2240	1.67	0.60

[a]The regression curves were obtained by plotting molar concentration (x) vs. peak area (y). [b]RRf is UV relative response factor determined in reference to pholcodine at 280 nm. [c]Cf is correction factor for the quantitation of the alkaloids at 280nm.

alyze two pure compounds in defined quantities under the same detection conditions and calculate ratio of slopes of the linear regression curves. Unless otherwise indicated, the regression analysis is done with the mass concentration of the compounds. Since the mass concentration does not include the exact amount of the component that enters the detector, more appropriate way for performing the regression analysis is with a series of molar concentrations. Linear calibration curves for all seven compounds were constructed using the peak areas and analyte's mass concentrations in the range of 1–150% (w/w) of the working concen-

tration. The linearity data using mass and molar concentration of all analytes were summarized in Table 1 and 2, respectively. The UV relative response factor (RRf) was determined as the ratio of slope of the regression line for each alkaloid and pholcodine.

Another approach for determination of RRf is to determine the detector sensitivity, defined as absolute response factor (signal output per unit concentration of a substance in the mobile phase entering the detector) (Ph.Eur.2.2.46). The relative response factor is expressed as the sensitivity of a detector for a given substance relative to a stan-

Table 3. Detector sensitivity and UV relative response factors of opium alkaloids

Compound	n (nmol)	ARf[a]	RRf[b]	Cf[c]
Morphine	1.402	509.8	0.88	1.14
Oripavine	0.336	2803.8	4.84	0.21
Codeine	0.668	596.3	1.03	0.97
Pholcodine	1.005	578.90	1.00	1.00
Papaverine	0.295	2577.5	4.45	0.22
Thebaine	0.321	2699.3	4.66	0.21
Noscapine	0.484	1019.2	1.76	0.57

[a]ARf is an absolute response factor determined as ratio between the signal output (mAU*s) measured at 280 nm and the amount of each compound (nmol) in the defined injection volume. [b]RRf is UV relative response factor determined as ratio of absolute response factors of each compound and the reference compound. [c]Cf is correction factor for the quantitation of the alkaloids at 280nm.

Table 4. Statistical comparison between the relative response factors of the target alkaloids, determined by: 'regression analysis/mass concentration' (RRf1), 'regression analysis/molar concentration' (RRf2) and 'detector sensitivity' approach (RRf3)

	RRf 1	RRf 2	RRf 3	d(RRf1/RRf2)[a]	d(RRf2/RRf3)[a]	d(RRf1/RRf3)[a]
Morphine	1.23	0.83	0.88	47.13	5.68	39.21
Oripavine	6.49	4.67	4.84	39.15	3.79	34.07
Codeine	1.38	1.00	1.03	37.77	3.00	33.75
Papaverine	5.21	4.25	4.45	22.67	4.76	17.09
Thebaine	5.99	4.50	4.66	33.06	3.62	28.42
Noscapine	1.70	1.67	1.76	1.73	5.64	-3.69

[a]$d = (RRf_{ni} - RRf_{nt})/RRf_{nt} \times 100\%$, p=0.05, DF=1, ($t_{tab}$=6.314)

dard substance, i.e. ratio of absolute response factors of the substance and the standard (Table 3). The results for the detector sensitivity, defined as absolute response factor (ARf), determined for each compound are summarized in Table 3. The RRf was expressed as a ratio of ARf of each compound and pholcodine. This approach gave more accurate RRf values than the conventional (mass concentration) regression analysis, because the calculation includes the exact amount of the substance that enters in the detector. However, unlike the 'regression analysis/molar concentration' approach, the calculation for 'detector sensitivity' approach includes only one concentration of the analyte, which is the major disadvantage of this approach.

For all the compounds having RRf values outside the range 0.8-1.2, a correction factor should be applied in the quantification (Ph.Eur.2.2.46). The correction factors (Cf) were calculated as a reciprocal of the relative response factor and are presented in the final columns of the Tables: 1, 2 and 3.

The different approaches used for determination of relative UV response factors (RRFs) of morphine, codeine, thebaine, oripavine, noscapine and papaverine by HPLC-DAD showed different values for the RRf. The statistical comparison between the RRf values obtained from different approaches (Table 4) showed that the difference between the 'regression molar concentration' and 'detector sensitivity' approaches can be considered as insignificant

and is due to the differences of the statistical calculation (five vs. one concentration point). The significant statistical difference between the 'regression mass concentration' approach and the other two approaches is an indicator that misjudgment can be made if the correction factor is calculated using the mass concentration of the standards. The most accurate way to determine RRf values is to calculate ratio of slopes of the linear calibration curves obtained from series of molar concentrations, because in the calculation is included the exact amount of the substance that enters in the detector. For the accurate determination of the content of alkaloids is necessary to include a correction factor (Cf), given in the last column of Table 2.

Conclusion

Different approaches for the determination of relative UV response factors (RRf) of morphine, codeine, thebaine, oripavine, noscapine and papaverine by HPLC-DAD, using pholcodine as the reference compound, were presented.

The 'regression analysis/molar concentration' approach gives the accurate approximation of the exact amount of the substance that enters in the detector and the calculation includes statistically enough concentration points (at least five), which makes this approach most advantageous one. The correction factor to be included for alkaloid determi-

nation is expressed as the reciprocal value of the factor of relative detector response calculated by regression analysis of dependence between the detector response and molar concentration of the alkaloids.

This method is suitable for rapid, routine analysis for the content determination of six alkaloids in standardised opium dry extract, raw opium and standardised opium tincture, through relative response factors of the opium alkaloids.

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Резиме

Определување на фактор на релативен одговор на детекторот на опиумските алкалоиди со примена на HPLC-DAD

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Клучни зборови: HPLC-DAD, Фактор на релативен одговор на детекторот (RRF), опиумови алкалоиди, внатрешен стандард

Во рамки на овој труд, претставен е соодветен метод за определување на фактор на релативен одговор на UV детектор (RRF) на морфин, кодеин, тебаин, орипавин, папаверин и носкапин со примена на високо-перформансна течна хроматографија (HPLC) и детектор со диодна решетка (DAD). Како референтна компонента за определување на RRF вредностите на алкалоидите беше избран фолкодин, стандардна супстанција.

Разделувањето на сите седум соединенија е постигнато со оптимизирано градентно елуирање со висока pH вредност на мобилната фаза, на реверзно-фазна колона со специфично обработена стационарна фаза (технологија на двојно C18-C18 сврзување). RRF вредностите на алкалоидите се утврдени со три различни пристапи: „регресиона анализа / масена концентрација", „регресиона анализа / молска концентрација" и „осетливост на детекторот".

Пристапот „регресиона анализа / молска концентрација" е најсоодветен за определување на RRF вредностите на алкалоидите, затоа што на овој начин се добива точна апроксимација за концентрација на аналитот што влегува во детекторот, а пресметката е статистички релевантна со оглед дека вклучува неколку различни концентрации (најмалку пет) на аналитот.

Предложениот метод е соодветен за проценка на квалитетот на стандардизиран сув екстракт од опиум, сиров опиум и стандардизирана тунктура од опиум, преку квантитативна анализа не само на морфин и кодеин, како што е наведено во соодветните монографии на Европската фармакопеја, туку и определување на главните онечистувања што потекнуваат од афион *Papaver somniferum* L. (Papaveraceae).