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Impurity profiling of morphine by liquid chromatography-heated electrospray ionization mass spectrometry (LC-HESI-MS)

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Abstract: Separation of morphine and its impurities (related substances specified in relevant European Pharmacopoeia monographs, as well as the other naturally occurring coextracted alkaloids) was obtained within a close retention window on a reverse phase HPLC column, eluted with optimized gradient, consisting of methanol and 5 mM ammonium formate, adjusted to pH 10.2. Systematic optimization of the chromatographic conditions was carried out using design of experiments. According to their mass spectra, known and specified impurities were identified and tentative structures for unknown impurities were proposed. MS analyses were performed in positive ionization mode using heated electrospray ionization. Fragmentation patterns of the eluted compounds under optimized mass spectrometry conditions indicated that all detected impurities are structurally related to morphine.

Key words: Impurity profiling, LC-DAD-HESI-MS^n, morphine, design of experiments

1. Introduction

Impurity profiling is strongly related to detecting, understanding the mechanism of action, and preventing side effects of medicines; therefore, analytical monitoring of impurities in active pharmaceutical ingredients (APIs) is a key component of drug development.[1,2] This indicates that the quality control of APIs should always be supported by additional purity profiling, based on the information obtained from the manufacturing process.[3] Furthermore, the impurity profiling represents part of an analytical strategy used for combating falsification of medicines.[4–6] Our previous work demonstrated that profound understanding of impurity profile is the key to a better understanding of the origin and differentiation of API samples.[7–9]

Considering the fact that morphine is isolated from a natural product, the profile of impurities largely encompasses organic compounds with similar structures. It is very likely that the most abundant poppy alkaloids would be coextracted under extraction conditions of morphine. Organic impurities with similar structure to morphine can be characterized using HPLC, as suggested in the European Pharmacopoeia (Ph.Eur).[10] However, the list of specified impurities does not indicate other alkaloids (e.g., thebaine, noscapine, papaverine), their biosynthetic intermediates, degradation products, and other classes of alkaloids. Since HPLC retention times

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may vary, uncertainty can arise as to whether a peak in a new retention time means a new impurity. Therefore, when impurity standards are not available, the method should provide online characterization, such as mass spectrometry (MS), as a detection tool in further determination of the unknown and unspecified impurities.

This work attempts to assess the ability of LC-MS^n to generate characteristic impurity fragmentation pathways, composed of a molecular ion mass, plus at least three fragmentation steps.[11,12] Our previous research on API authentication and prevention of falsification, based on the impurities detected in morphine samples[7] and assessment of the qualitative profile of alkaloids in poppy straw,[13–15] pointed out the necessity of online characterization of the present analytes. Thus the aim of this part of the research was to advance LC method identification performances of a suitable 'MS friendly' HPLC method for separation and identification of morphine and its known and unknown related substances and possible degradation products, using a well-designed strategy for optimization and robustness verification.[16–19]

2. Results and discussion

2.1. Optimization of the chromatographic conditions

In order to optimize the chromatographic conditions, 2^3 central composite face centered design of experiments (CCFC DoE) was employed, which involved a set of 11 planned experiments, and investigation of two experimental factors: buffer concentration (x1), in a range from 1 to 10 mM, and buffer pH value (x2), in a range from 9.0 to 10.2. The chromatographic response was assessed through the critical resolutions (between morphine and impurity E and between morphine and impurity B) and the retention factor of impurity F. The results obtained during the method optimization are summarized in Table 1. The effects of the different experimental conditions on the defined chromatographic responses are shown through the normalized coefficients of the CCFC design (Figure 1). The alteration of the chromatographic responses over the defined area of the experimental factors was assessed using response surface methodology (RSM) (Figure 2).

Table 1. Experimental data for assessment of the chromatographic response during the method optimization.

Experiment no.	x_1 (buffer conc., mM)	x_2 (buffer pH)	Rs1 (imp.E/MO)	Rs2 (MO/Imp.B)	k' (imp.F)
N1	1	9	1.35	0.0	1.2
N2	10	9	1.81	5.0	1.2
N3	1	10.2	4.56	0.0	0.6
N4	10	10.2	5.93	9.4	0.7
N5	1	9.6	2.05	0.0	0.9
N6	10	9.6	3.46	5.3	1.1
N7	5.5	9	2.01	4.0	0.9
N8	5.5	10.2	5.81	10.7	0.7
N9	5.5	9.6	3.26	6.1	1.1
N10	5.5	9.6	3.26	6.1	1.1
N11	5.5	9.6	3.22	6.1	1.1

The results of the chemometric experiments showed that all factors affected the chromatographic parameters. Critical resolution between impurity E and morphine was perceived, and impurity F was eluted in the column void time (1.4 min). By increasing the buffer concentration and the pH of the buffer, impurity E/morphine resolution (Figure 2a) and impurity B/morphine resolution (Figure 2b) was enhanced. The retention factor (k' value) for the first eluted peak (impurity F) was increased (above 1, which was set as target minimum value) by increasing the buffer concentration and decreasing the buffer pH value (Figure 2c). However,

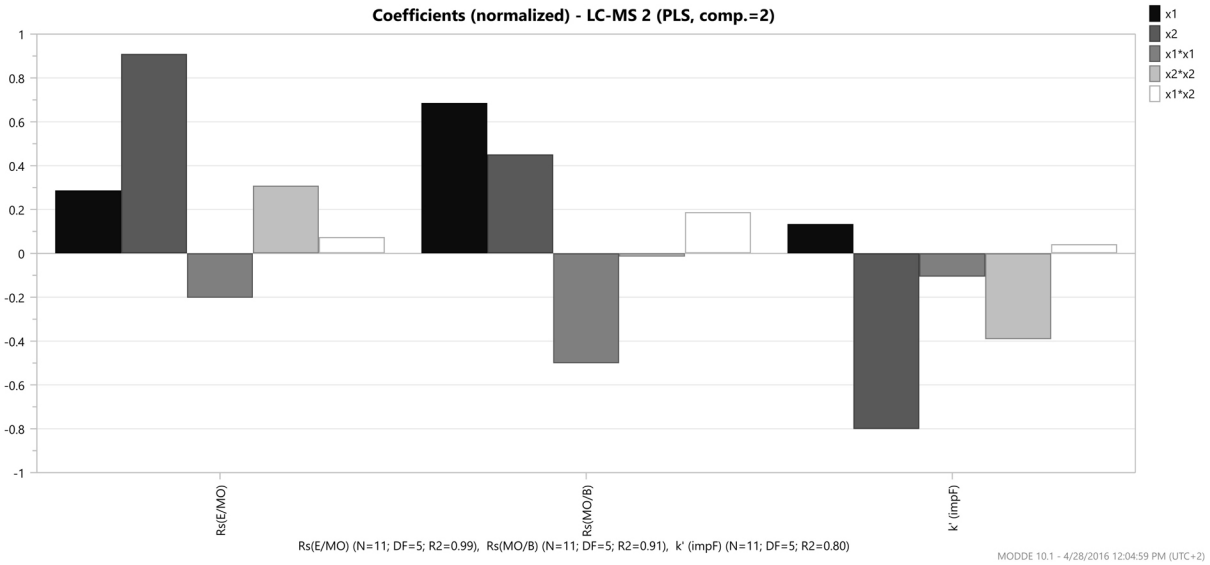

Figure 1. Normalized coefficients of the 2^2 CCFC DoE showing the effects of different chromatographic conditions: b1 (buffer concentration), b2 (buffer pH value) and their interactions (b11, b22, b12) on the chromatographic descriptors (resolution between critical pairs of analytes and retention factors of the first eluting peak) during the method optimization by use of chemometrics.

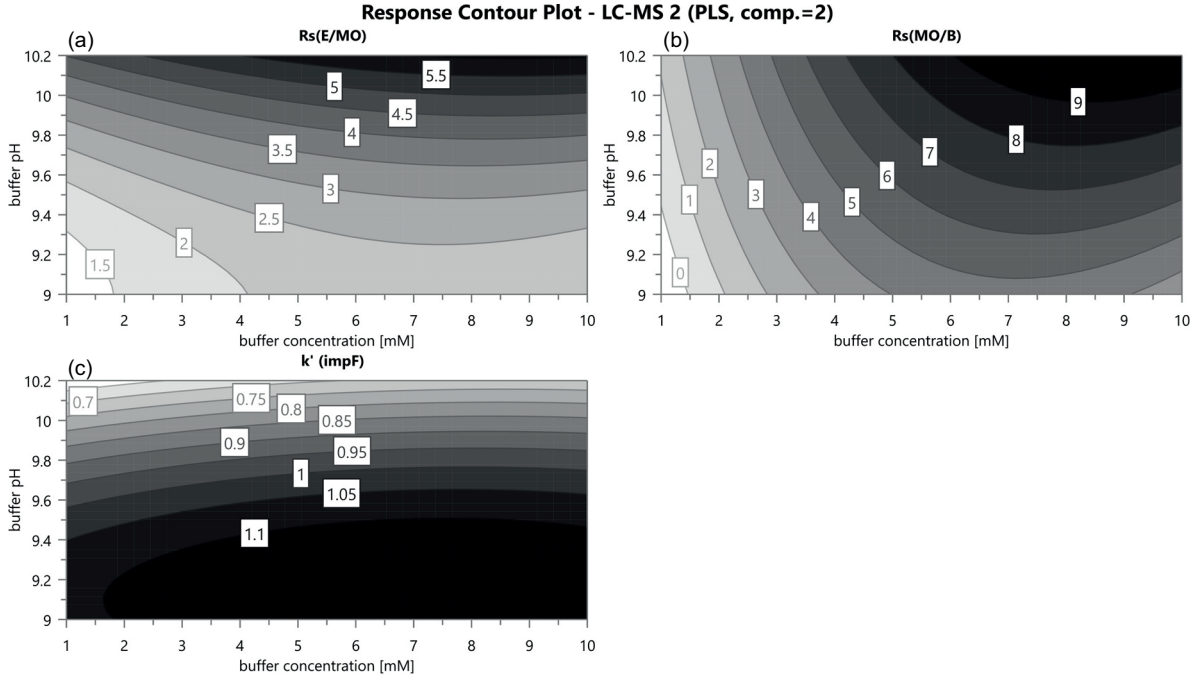

Figure 2. Contour diagram presenting the effect of the experimental factors; buffer concentration (x1) and buffer pH value (x2) on the critical resolutions: a) impurity E/MO (y1), b) MO/impurity B (y2), and c) retention factor of impurity F (y3).

the lowest possible buffer concentration is desirable for better sensitivity of the mass spectrometer. As for the pH value, attention should be paid to the column stability. According to the RSM results shown in Figure 3, the chromatographic conditions were optimized. Since the operational pH range of the column was to 11.5, the

maximal value was chosen (10.2), considering also the buffer capacity. For the buffer concentration, a mid value was chosen, since both critical resolutions were above the target value of 2.0 (Figures 3a and 3b). As for the retention factor of impurity F, the content of organic solvent (MeOH) was decreased in order to keep the value above 1.0 (Figure 3c).

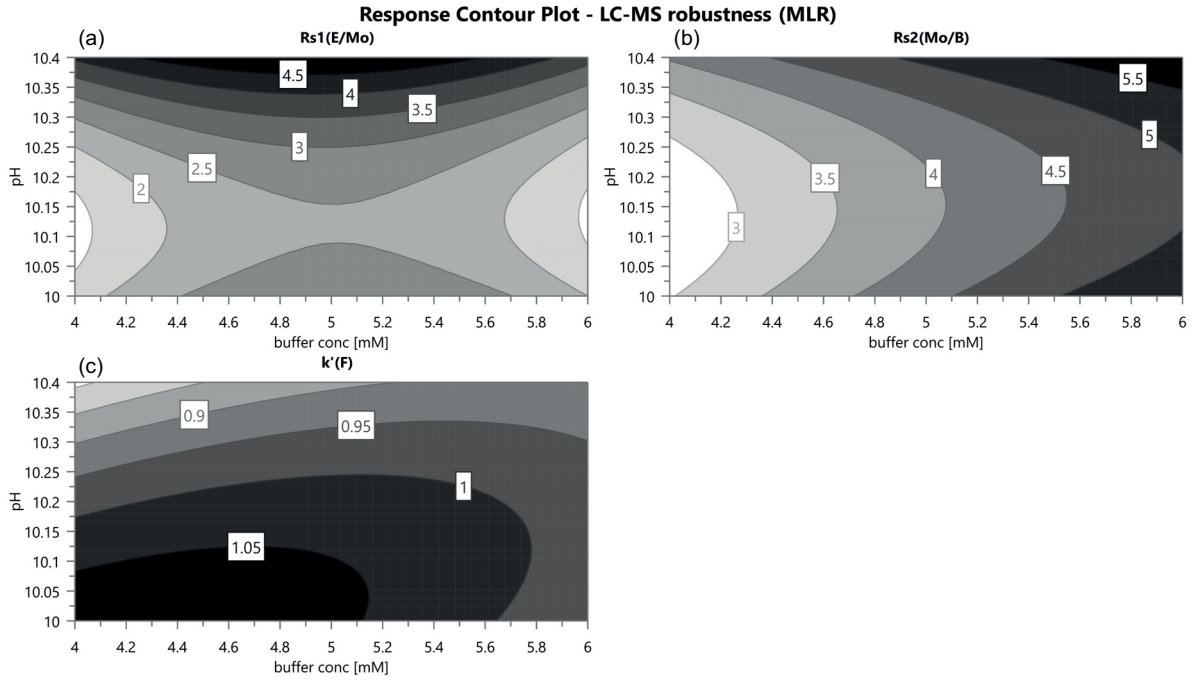

Figure 3. Results obtained from testing the robustness of the methods (2^2 Full Factorial Quadratic Design of Experiment) - The effects of changes in different analytical conditions during the robustness testing of the method; x_1 – buffer concentration (4–6 mM) and x_2 – buffer pH (10.0–10.4) on the chromatographic response: a) y_2 – resolution between impurity E/morphine, b) y_3 – resolution between morphine/impurity B and c) y_1 – retention factor (impurity F).

2.2. Method validation

Under the proposed chromatographic conditions all analytes were completely separated (Figure 4). Results from method validation, in accordance with International Conference on Harmonization (ICH) guidelines Q2 R1[20] showed satisfactory specificity, linearity, sensitivity, precision, accuracy, and robustness. The number of theoretical plates (N) for all analytes (N > 5000) indicates a good separation efficiency of the applied column. Capacity factor values for all peaks were between 2 and 10. All peaks have good shape, with symmetry factor (As) values between 0.8 and 1.2.

The linearity of the method was demonstrated for morphine reference solutions ranging from 1.275 to 11.475 μg/mL (0.05%–0.45% of the morphine working concentration). The results obtained from the regression analysis of the peak area (y) versus concentration (x) data indicated that the method was linear (y = 13.931x – 3.8973, R^2 = 0.9999).

The limit of detection (LOD) and limit of quantification (LOQ) were determined from the residual standard deviation of the regression line (σ) and the slope (S) in the concentration range from 1.275 to 11.475 μg/mL (0.05%–0.45% of the morphine working concentration). The LOD for morphine (3.3 σ/S) is 0.15 μg/mL (0.006% of the morphine working concentration), which is within the acceptance criteria (LOD was $\leq$0.05%

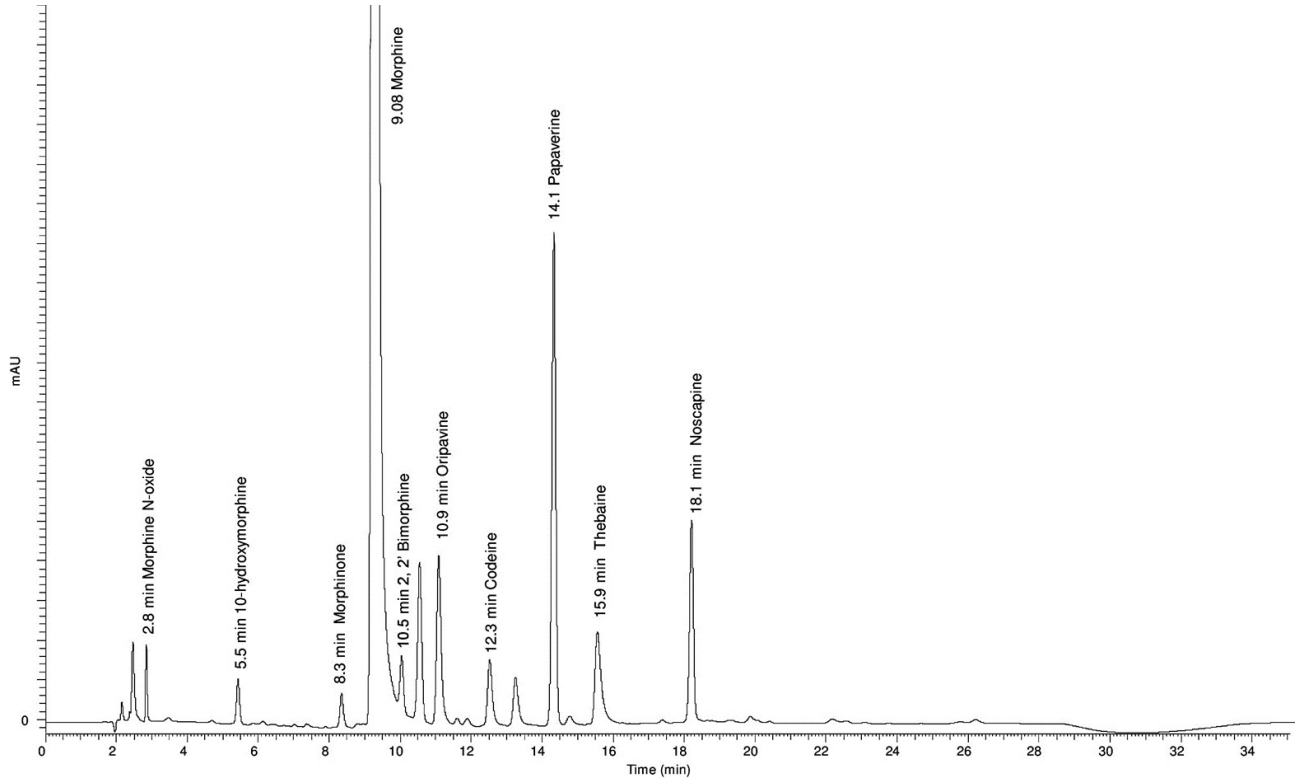

Figure 4. UV chromatogram of morphine and its known impurities.

of the morphine working concentration (Ph.Eur disregard limit (10)), i.e. $\leq$1.275 μg/mL). The LOQ value (10 σ/S) was 0.44 μg/mL (0.017% of the morphine working concentration), which is much less than level of specification (0.2% of the morphine working concentration).

The repeatability was performed by analyzing six replicate injections of an impurity standard solution at 100% of the specified limit with respect to the working strength of API (0.2% of the morphine working concentration for each impurity, except for impurity B: 0.4% of the morphine working concentration). The percentage relative standard deviations of peak areas (n = 6) were less than 2.0% for each impurity, which indicated acceptable repeatability of the method. Additionally, precision at LOQ was performed by analyzing three replicate injections of a standard solution of morphine at LOQ level and the percentage relative standard deviation was less than 5.0%.

The accuracy of the method was assessed by comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, i.e. method for related substances described in Ph.Eur monographs on morphine hydrochloride trihydrate (04/2008:0097) and morphine sulfate pentahydrate (04/2008:1244), since the impurity and degradation product standards are unavailable. The bias for individual, unknown impurities obtained by the two methods was not more than the acceptance criterion of $\pm$0.03 and the bias for total impurities was within the acceptance criterion of $\pm$0.10.

The amount of each impurity [% m/m] present in the test solution (morphine concentration about 2.5 mg/mL) was calculated using the peak due to morphine obtained with reference solution (diluted test solution to about 0.005 mg/mL, i.e. 0.2% solution relative to test solution) as external reference. For the purpose of the study (API fingerprinting) no peak was disregarded.

2.3. Robustness validation

Within method validation, suitability of the chromatographic system was demonstrated using mixed standard solution, containing related substances specified in the Ph.Eur monographs on morphine hydrochloride trihydrate (04/2008:0097) and morphine sulfate pentahydrate (04/2008:1244):[10] impurity A (codeine), impurity B (2,2'-bimorphine), impurity C (oripavine), impurity D (10S-hydroxymorphine), impurity E (morphinone), and impurity F (morphine N-oxide), as well as other naturally occurring coextracted (commercially available) alkaloids: thebaine, noscapine, and papaverine.

Specificity was demonstrated by showing that the peaks were acceptably resolved, by performing peak purity tests using diode array detector and mass spectrometry. Additionally, specificity was proved by comparing the impurity profiles of API samples obtained with the pharmacopeial method for determination of related substances of morphine, described in the relevant Ph.Eur monographs.

Since robustness testing of analytical methods[20] requires extensive experimental work (where intentional changes in experimental conditions are made and studied), here an accurate and rational assessment of the robustness of the method was done by use of chemometrics. Minor changes in experimental factors values were made by performing a set of 12 experiments defined by the 2^2 full factorial quadratic design of experiments (Table 2). The following method parameters were altered in order to test the robustness: buffer concentration (x_1), in a range ± 1 mM from the proposed concentration of 5 mM (corresponding to maximum 20% variation of the system suitability criteria[21,22]) and buffer pH (x_2), in a range ± 0.2 pH value from the proposed pH $=10.2$ (corresponding to recommended maximum variation of pH.[20]) These method parameters were determined as critical during method optimization by use of chemometrics. System suitability solutions were analyzed to assess if these changes had a significant effect on the defined method parameters. The chromatographic response was assessed through the critical resolutions (between morphine and impurity E and morphine and impurity B) and the retention factor of impurity F.

System suitability criteria for the method should be fulfilled, i.e. the critical resolutions between morphine and impurity E and morphine and impurity B should be above 2.0 and the retention factor of impurity F should be above 1.0. Table 2 and Figure 4 summarize the results of the robustness study. The small changes in buffer concentration (± 1 mM) and pH of the mobile phase ($\pm$ 0.2) did not change significantly the chromatographic responses defined within the acceptance criteria (Figure 3). The critical resolutions between morphine and impurity E and morphine and impurity B remained above 2.0 (Figures 3a and 3b) and the retention factor of impurity F was around the defined value of 1.0 (Figure 3c). However, the retention factor of impurity F was found to be affected at low buffer concentration (4 mM) and high pH values (pH 10.4), especially when the experimental factors were changed simultaneously. Therefore, it is recommended to maintain buffer pH at 10.2 $\pm$ 0.05 and buffer concentration at 5 $\pm$ 0.5 mM.

2.4. Identification of morphine impurities using mass spectrometry

The starting material and known impurities under investigation are alkaloids containing basic nitrogen, which makes them preordained for electrospray ionization in positive ionization mode. In order to test mass spectrometer selectivity, this method was optimized with analysis of mixed standard solution containing all pharmacopeia impurities and related substances listed in Table 3: morphine N-oxide (impurity F), 10-S-hydoxymorphine (impurity D), morphinone (impurity E), 2,2' bimorphine (impurity B), oripavine (impurity C), codeine (impurity A), papaverine, phebaine, and noscapine. Figure 4 shows a UV chromatogram of all the impurities present in mixed standard solution.

Table 2. Summary of data for robustness study parameters altered to test method robustness, using the 2^2 full factorial quadratic design of experiments.

Experiment	Parameters of the method (factors)		Chromatographic descriptors (responses)		
	x_1(mM)	x_2(pH)	k'(imp.F)	Rs1(Imp.E/ morphine)	Rs2(morphine/ Imp.B)
N1	4	10.0	1.09	2.09	2.32
N2	5	10.0	1.03	2.15	5.57
N3	6	10.0	0.97	2.44	4.96
N4	4	10.2	0.97	2.69	3.28
N5	5	10.2	1.03	2.21	3.74
N6	6	10.2	0.95	2.46	5.30
N7	4	10.4	0.79	2.68	4.16
N8	5	10.4	0.88	7.73	4.83
N9	6	10.4	0.91	2.40	6.11
N10	5	10.2	1.03	2.18	3.74
N11	5	10.2	1.03	2.19	3.72
N12	5	10.2	1.03	2.13	3.71

Table 3. Fragments, retention times, and relative retention times of known impurities, analyzed with the LC-MS method.

Peak name	MW	r_t	RRt	λ_{max}	MH^+	MS^2	MS^3
Morphine *N*-oxide (Imp F)	**301**	2.8	0.31	202; 212; 292	**302**	**284**; 267	174; **201**; 211; 215; 229; 268
10-*S*-hydoxymorphine (Imp D)	**301**	5.5	0.60	224; 260	**302**	201; **227**; 284	**162**; 201; 211; 215; 229; 268
Morphinone (imp E)	**283**	8.3	0.90	236; 272; 348; 372	**284**	**227**; 284	**209**
Morphine	**285**	9.2	1.00	218; 236; 286	**286**	**201**; 211; 215; 229; 268	**173**; 155; 165; 237;193; 185
2,2' Bimorphine (imp B)	**568**	10.5	1.14	242; 314; 370	**569, 286**	**551**	**437**
Oripavine (imp C)	**297**	10.9	1.19	228; 286; 352	**298**	**267**	**249**
Codeine (imp A)	**299**	12.3	1.34	222; 284	**300**	**215**; 243, 282	**182**
Papaverine	**339**	14.1	1.53	238; 278; 341	**340**	**202**;	170
Thebaine	**311**	15.3	1.66	232; 284	**312**	**281**; 249	**249**; 266
Noscapine	**413**	17.9	1.95	220; 292; 312	**414**	220	204

MW– molecular weight; r_t – retention time; RRt – relative retention time; MH^+ – parent ion m/z; MS^2, MS^3 – fragments, most abundant ions are marked in bold

The first challenge for this mass spectrometry method was to distinguish morphine *N*-oxide (impurity F) from 10-*S*-hydoxymorphine (impurity D), which are structural isomers with molar mass 301. Namely, impurity D differs from morphine by one hydroxyl group attached to position C-10 on morphine B ring opposite from impurity F, which includes one oxygen atom attached to nitrogen (position 17) and forms morphine N-oxide.

According to the obtained mass spectra from experimental data, impurity F (molecular weight 301, $[M + H]^+ = 302$), as unstable morphine N-oxide radical, immediately transforms to m/z 285 (most abundant peak in MS^2), followed by loss of 16 amu, which implies loss of oxygen. The presence of m/z 268 in impurity F's MS^2 spectra indicates loss of 35 amu due to morphine ring E opening and detaching of the CH_3NCH_2-group.

Mass spectra obtained for impurity D (molecular weigh 301, $[M + H]^+ = 302$) show the most abundant mass spectra m/z 227 due to loss of 75 amu (loss of CH_3 and morphine ring D). Unlike impurity F, ion m/z 284 is present here, which indicates loss of 18 amu (H_2O) from the parent compound. Both impurities in their MS^3 spectra show fragments 162 and 201, as part of morphine fragmentation. This indicates that although impurities F and D are structural isomers they can be distinguished by their fragmentation pathways. In the absence of specific standards for these impurities, for the MS part of this method information from mass spectra was used as a verification tool and it was defined that impurity F occurs at a retention time of about 2.83 and impurity D occurs at a retention time of about 5.4 min.

Additionally, morphine base (MoB), morphine sulfate (MoS), and morphine hydrochloride (MoH) samples were analyzed and effort was made to identify the following unknown impurities/morphine related products and keeping in mind the mass spectrometry results, the most probable structures of these impurities were suggested. All discussed fragments of unknown impurities are summarized in Table 4.

Table 4. Fragments, retention times, and relative retention times of unknown impurities found with LC-MS method in 14 different morphine samples.[7]

Peak name	r_t	RRt	λ_{max}	MH^+	MS^2	MS^3
Impurity X1	6.2	0.68	220; 232; 280	**344**	**328**	211; 225; 237; 250; 268; **286**;
Impurity X2	6.9	0.75	220; 232; 278	**388**	**372**; 344	211; 225; 237; 250; 268; **286**;
Impurity X3	7.1	0.78	235; 268	**286, 476**	**459**; 415	**176;** 371
Impurity X4	7.8	0.85	235; 268	**520; 286**	**503**; 269; 520	**176**; 162
Impurity X5	10.8	1.18	235; 268	**569, 286**	**551**	**437**
Impurity X6	11.4	1.24	218; 236; 286	**284**	**227**; 284	**209**
Impurity X7	11.6	1.27	218; 236; 286	**284**	**227**; 284	**209**
Impurity X8	11.7	1.28	218; 236; 286	**284**	**227**; 284	**209**
Impurity X9	10.4	1.14	218; 236; 286	**358**	201; **286**; 229; 268	**174**; 162
Impurity X10	13.6	1.48	242; 314; 370	**572**	201; **286**; 229; 268	**174**; 162
Impurity X11	14.0	1.53	242; 314; 370	**572**	201; **286**; 229; 268	**174**; 162
Impurity X12	14.4	1.57	242; 314; 370	**572**	201; **286**; 229; 268	**174**; 162
Impurity X13	7.5	0.82	220; 232; 280	**327**	**284**; 133	**133**

MW– molecular weight; r_t – retention time; RRt – relative retention time; MH^+ – parent ion m/z; MS^2, MS^3 – fragments, most abundant ions are marked in bold

Unknown impurity X1 with RRt = 0.68 shows a molecular ion peak with m/z 344, which in the second step of fragmentation indicates clear MS^2 spectra where the only present peak is m/z 328 (loss of 17 amu, which indicates on hydroxyl group). This peak m/z 328 indicates a well-known morphine-related compound monoacetylmorphine that gives rise to product ions similar to those of protonated morphine. The ion at m/z 286 is formed by deacetylation (loss of ketene) from m/z 328. The base peak at m/z 268 can be produced either by direct loss of acetic acid from m/z 328 or by loss of H_2O from m/z 286. The MS^3 spectrum of m/z 268 results in product ions at m/z 250 (268- H_2O), 237, 225, and 211 formed by partial cleavage of the piperidine ring, as described by Zhang et al.[23]

As additional confirmation of the X1 structure, unknown impurity X2 structure was suggested. This impurity at RRt = 0.75 has a molecular ion peak m/z 388, which points to the morphine-related structure diacethyloxymorphine. Furthermore, this structure shows fragments m/z 344, which indicates the monoacethyl-

morphie structure and m/z 372 as the most abundant peak in the second step of fragmentation, explained as a result of loss of 17 amu (hydroxyl group). MS^3 spectra from the parent compound m/z 388 present three interesting peaks, such as m/z 328, previously explained as product of 344; m/z 250, 237, 225, and 211, which points to a morphine nucleus as in structure X1.

Impurity marked with X3 at RRt = 0.78 points to morphine due to the presence of ion m/z 286 and ion m/z 476 in the full mass spectra. Most probably m/z 476 (difference from bimorphine 93 amu) is a bimorphine-like structure, consisting of one morphine core attached to A, B, and D morphine rings, as residuals from the second morphine. The second step of fragmentation points to a loss of hydroxyl group to form ion m/z 459, which in the third step of fragmentation undergoes cleavage of one CH_3CH_2NH- group from one morphine E ring (loss of 44 amu) to form m/z 415 and also cleavage of two CH_3CH_2NH- groups (loss of 88 amu) to form m/z 371. The most abundant peak in the MS^3 spectra is ion m/z 176, which indicates loss of 286 amu and confirms the suggested structure.

At RRt = 0.85 unknown impurity X4 with m/z 520 was investigated. The obtained MS^3 spectra of this impurity confirms m/z 176 ion (loss of morphine structure 286 amu) as well as all ions discussed in the MS^3 spectra of impurity X3. The first step of fragmentation of X4 also indicates a bimorphine-like structure, whose most abundant ions are m/z 520 and m/z 286, followed by loss of 17 amu (hydroxyl group) in MS^2, confirmed by peaks m/z 503 and m/z 269, respectively. The suggested structure of this impurity differs from a bimorphine structure by 48 amu. These observations lead to the conclusion that this impurity with m/z 520 most probably consists of two morphine structures, one with detached CH_3CH_2NH- group and saturated C-C bonds on both morphine B rings.

Impurity X5 at RRt = 1.18 (which is very close retention time to the main compound, morphine) shows the same full mass spectra and fragments as morphine. The fragmentation of morphine and most abundant ions are explained above and confirmed by the literature.[24–30] Difference in retention time of morphine and unknown impurity X5 (higher retention) indicates that the unknown compound is less polar than morphine, which most probably originates from different arrangement of hydroxyl groups or substitution of less polar groups with the same mass as OH groups on morphine rings B and A. All of the above-mentioned information suggests that morphine and impurity X5 are structural isomers with the same molar mass; the same fragments but different polarity equals different retention time.

A group of impurities at RRt 1.24 (X6), 1.27 (X7), and 1.28 (X8) points to dihydromorphone-like structures or, in other words, dihydromorphone structural isomers, with the same m/z 284 (dihydromorphone molecular mass is 283) and the same fragmentation until MS^3 but different polarity due to different arrangement of the same substituent, which in this case can only be different substitution of the –OH group on the dihydomorphone structure.

Unknown impurity X9 at RRt 1.14, present only in one MoB sample,[7] shows mass spectra with m/z 358. In MS^2 this impurity is followed by loss of 72 amu to form only one most abundant peak ion m/z 286 (morphine), which is followed by fragmentation, the same as morphine fragmentation in MS^3. The most probable structure of this impurity is morphine with substituent of 72 amu (CH3-CH-CH_2CH_2OH) on position 2 on morphine A ring.

Peaks at RRt 1.48, 1.53, and 1.57 (X10, X11, and X12) are probably bimorphine-like structures with saturated double bonds on morphine rings A or C. These three impurities in the full mass spectra range show m/z 572, which is a difference of only one saturated double bond from the bimorphine original structure. It is

assumed that this saturation influences the retention time (different from all other bimorphine-like structures discussed above). As all previously discussed bimorphines, in the first fragmentation step all of the three compounds lose 17 amu, followed by loss of 286 to form m/z 176. Unknown structures X10-12 differ in intensity abundance of ion peaks in MS^2 and therefore it can be concluded that they are structural isomers.

Unknown impurity X13 with RRt = 0.82 shows a molecular ion peak with m/z 327, which in MS^2 shows fragments, such as m/z 133, which is probably the most stable metoxybenzyl radical (formed with loss of 194 amu) and peaks with m/z 284 as a result of a loss of 43 amu in the form of a $CH_3C{=}O$ group. The presence of metoxybenyl radical (note that monoacetyl group is attached on the morphine ring C) plays a crucial role in morphine structure fragmentation[21,22,30] and the mass spectra of this impurity indicate that this impurity is a monoacetylmorphine derivate.

In this work, a suitable "MS friendly" HPLC method for separation and identification of morphine and its known and unknown related substances and possible degradation products was developed and successfully employed for origin determination of morphine samples using pattern recognition techniques.[7] That part of the research proved the applicability of the method and highlighted its advantages over the existing pharmacopoeial HPLC method for determination of related substances of morphine,[10] regarding authentication and prevention of falsification.

The method for separation of morphine-related compounds proposed by Ph. Eur, using anionic ion-pairing agents at acidic pH to avoid peak tailing, is not convenient for coupling with mass spectrometer due to the nonvolatility of the ion-pair reagents. In order to avoid use of ion-pairing agents, we searched for an alternative mobile phase. Due to the basic nature of morphine impurities (morphinane core), good separation was obtained at alkaline pH, where the amine group would not be ionized. This is generally not possible with bonded silica-based columns because of the instability of the silica matrix at alkaline pH.[20–22] A reversed-phase columns with bidentate C18-C18 bonding technology that prevents interactions between noncharged basic compounds with the underlying silica at high pH was chosen as a stationary phase.[31] Some of the mobile phase buffer options for high pH included trifluoroacetic acid (TFA), trichloroacetic acid (TCA), ammonium acetate (AmAc), and ammonium formate (AmF) in combination with ammonia. TCA and AmAc did not provide a satisfactory solution, unlike TFA, but in this case the large ion reduced the sensitivity of the mass spectrometer. The optimal chromatographic and mass spectrometry conditions were obtained using AmF in combination with ammonia for pH adjustment. As for the choice of a UV wavelength, although morphine-related compounds have absorption maxima around 283 nm, 230 nm was used for detection in order to expose morphine N-oxides. An additional step of heating the mobile phase and sample mixture prior to ionization (heated electrospray ionization (HESI)) was used to maintain a high flow rate of the mobile phase of 1 mL/min in order to achieve faster analysis time and at the same time accomplish vaporization of the mobile phase before ionization. During method optimization, both ESI and HESI modes were tested and it was concluded that the additional heating of the probe does not induce additional degradation of morphine.

Using DoE results, the separation of all the impurities was obtained with an optimized gradient elution with methanol and 5 mM ammonium formate, pH adjusted to 10.2. All compounds were fully resolved and eluted within 20 min and satisfactory ionization and mass spectrometer sensitivity were achieved. After method validation, closer examination of the results obtained from mixed standard solutions presented in Table 3 suggests that all morphine-related compounds undergo similar fragmentation patterns as morphine, after their side chains or function groups detach. In previous years, soft ionization methods were intensively used for investigation of morphine and its metabolites,[24–34] versus the electron impact (EI) mass spectra of morphine

and morphine-related compounds, which were elaborated at the end of the last century.[24–27] The results obtained from comparative study of an ion trap mass spectrometer and a triple quadrupole system revealed that the MS^2 spectrum of morphine (m/z 286) contains major product ions at m/z 268, 229, 211, and 201, and minor ions at m/z 237, 193, 185, 173, 165, and 155. The base peak at m/z 201 is derived from m/z 286 by partial cleavage of the piperidine ring and consecutive losses of an amine ($CH_2CHNHCH_3$) and CO.[27,28] Since a detailed explanation of the fragmentation of morphine, codeine, thebaine, and oripavine has been presented previously,[23] only the unspecified impurities are discussed in the section results.

3. Experimental

3.1. Chemicals and reagents

Morphine for system suitability CRS, containing Ph.Eur specified impurities B (2,2'-bimorphine), C (oripavine), E (morphinone), and F (morphine N-oxide), was purchased from EDQM. Working standards for Ph.Eur impurity A (codeine) and the naturally occurring alkaloids, thebaine, papaverine, and noscapine, as well as the samples, morphine hydrochloride trihydrate (MoH), morphine sulfate pentahydrate (MoS), and morphine base (MoB), were obtained from Alkaloid A.D. Skopje (Skopje, R. Macedonia).

Methanol (MeOH, HPLC grade), sodium heptanesulfonate (pa), and phosphoric acid (pa) were purchased from Merck, Darmstadt, Germany. Acetic acid (pa), ammonium formate (pro analysis), and formic acid (pa) were purchased from Sigma-Aldrich (Steinheim, Germany). Water (highly purified) was obtained with a TKA-LAB Reinstwasser system (Niederelbert, Germany).

3.2. Sample preparation

Mixed standard solution was prepared by dissolving 5 mg of morphine for system suitability CRS in MeOH, adding 100 μL of the corresponding methanol stock solution (concentration of 1 mg/mL) of codeine (Ph.Eur impurity A), thebaine, papaverine, and noscapine, and diluted to 2 mL with MeOH. Sample solution was prepared by dissolving about 0.125 g of the substance to be examined in MeOH and diluted to 50 mL with the same solution. Reference solution, used for the calculation of each impurity's content,[7] was prepared by dilution of 1.0 mL of the test solution to 100.0 mL with MeOH and further dilution of 2.0 mL of this solution to 10.0 mL with MeOH. All samples and standards were prepared in duplicate. Each sample preparation was injected twice in the HPLC system. Six replications of each quantification standard were injected on a daily basis to confirm repeatability of the HPLC system. In order to check the performance of the HPLC system, prior to every analysis system suitability solution containing all known impurities was analyzed. Standards, samples, and system suitability solutions used for method validation were prepared and injected as recommended by ICH guidelines Q2 R1.[20]

3.3. Instrumentation and LC-MS conditions

The analysis was performed on a Thermo Scientific Dionex Ultimate 3000 UHPLC-UV-DAD system consisting of a Thermo Scientific Dionex Ultimate 3000 pump, Thermo Scientific Dionex Ultimate 3000 injector, Thermo Scientific Dionex Ultimate 3000 Column Thermostat Compartment, and Thermo Scientific Dionex Ultimate 3000 diode array detector associated with a Thermo Scientific LTQ XL linear ion trap mass spectrometer equipped with a HESI probe and controlled by Chromeleon 5.0 and Xcalibur 2.0 software. The separation of the detected impurities was achieved on a Zorbax Extend C18 column (Agilent Technologies), 250 mm $\times$

4.6 mm i.d., and 5 μm particle size. Elution was performed at flow rate of 1.0 mL/min, using mobile phase consisting of MeOH and ammonium formate buffer (5 mM; pH 10.2 adjusted using 25% ammonia solution) with the following gradient: 0–1 min 15% MeOH; 1–5 min from 15% to 50% MeOH; 5–15 min from 50% to 75% MeOH; 15–20 min 75% MeOH; 20–25 min from 75% to 15% MeOH; 25–35 min 15% MeOH. The injection volume was 10 μL, the column temperature was maintained at 35 °C, and detection was carried out at 230 nm.

The ESI probe needle temperature was 350 °C, and the ion transfer tube was 325 °C under sheath gas (nitrogen) at 50 psi and auxiliary gas helium at 10 psi. Mass spectra were recorded in the range of 50–800 m/z, with maximum accumulation time of 500 ms. The collision-induced fragmentation was performed in an ion trap using helium as collision gas at 3.5 eV. The specified impurities were confirmed by retention times and UV spectra and all detected impurities (specified and unknown) were identified using their mass spectra and fragmentation patterns.

3.4. Chemometrics

MODDE 10.1 software for DoE and optimization (Umetrics, Umea, Sweden) was used for optimization of the experimental factors through an appropriate factorial design. The designed experiments were conducted on a mixed standard solution. DoE was used to assess the influence of the ionic strength and the pH of the mobile phase buffer on chromatographic responses (2^2 CCC DoE) and for the robustness testing during method validation (2^3 CCC DoE).

4. Conclusion

The discussed results obtained in this study demonstrate that the proposed LC-DAD-HESI-MS method used for impurity profiling of MoB, MoH, and MoS API samples represents a useful tool for online characterization of known and unknown morphine-related impurities and degradation products. This LC-MS^n method has the ability to generate characteristic impurity fragmentation patterns, which consequently can be used as an advanced tool for achieving improved identification performances. According to MS spectra, tentative structures for detected unknown impurities were proposed, confirming that all detected impurities are structurally related to morphine. Therefore, the suggested LC-MS method provides a broad 'analytical window' in relation to the identified impurities, which allows authenticity verification of APIs from various manufacturers.

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