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Development and validation of a liquid chromatography method for simultaneous determination of three process-related impurities: yeastolates, triton X-100 and methotrexate

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ABSTRACT

Yeastolates, triton X-100 (TX-100) and methotrexate (MTX) are common process-related impurities (PRI) in cell-based bioproduction of many active biopharmaceuticals. In this study, a reverse phase high performance liquid chromatography (RP-HPLC) method coupled with ultraviolet (UV) detection was developed for simultaneous determination and quantitation of these impurities. The chromatographic separation was achieved using a Jupiter C4 column and analyses of yeastolates, TX-100 and MTX were monitored at 257, 280 and 302 nm, respectively. The method was further validated with respect to selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy. The limits of quantitation for yeastolates, TX-100 and MTX were determined to be 27 ppm, 10 ppm and 41 ppb, respectively. Finally, the suitability of the method for analyses of recombinant human hyaluronidase (rHuPH20) in-process (viral inactivation, QFF, PS, APB and CHT filtered, final viral filtrate) and final manufacturing materials was demonstrated, and trace levels of yeastolates, TX-100 and MTX were reliably measured except for three matrices early in the purification process in which TX-100 was not accurately determined due to interfering effects.

1. Introduction

Therapeutic proteins represent an important and fast growing class of drug products on the current market. Many protein drug substances are produced in host cells as diverse as bacteria, yeast, insect or mammalian cells [1]. Among these, Chinese Hamster Ovary (CHO) cells are a very common cell substrate for biopharmaceutical products today [2]. For example, the recombinant human hyaluronidase (rHuPH20) drug product utilizes CHO cells to produce rHuPH20 drug substance [3]. During its manufacturing processes (Fig. 1), various ingredients are added to the cell culture medium to promote cellular growth and protein production. Yeastolates (also known as yeast extract) are among the most common nutrients because of their cost-effectiveness, serum-free nature, and proven beneficial effect on cell growth [4,5]. Methotrexate (MTX) is another important ingredient used in cell culture media for cell amplification in dihydrofolate reductase (DHFR) deficient CHO host cells [6]. After cell harvest and clarification, solvents and detergents such as tri(n-butyl) phosphate (TNBP) and triton X-100 (TX-100) are commonly added for viral inactivation and removal [7].

The assessment of these process-related impurities is one of the key considerations in process development studies. Due to potential antigenicity, immunogenicity or toxicity of some process-related impurities [8,9], they must be eliminated in downstream process steps. Purification steps are incorporated to minimize these impurities in the final drug substance. As shown in Fig. 1, the production of rHuPH20 drug substance utilizes a complex medium containing CD-CHO, CD-CHO AGT, glutamax, MTX, yeastolates, insulin, glucose and sodium butyrate, and employs four stages of column purification in the order of Q-Sepharose Fast Flow (QFF), Phenyl Sepharose (PS), Amino Phenyl Boronate (APB) and Ceramic Hydroxyapatite (CHT) to eliminate process-related impurities. In this study we will focus on three common process-related impurities; yeastolates, TX-100 and MTX. Unfortunately, there has been, to date, no published information about analytical quantitation of yeastolates. A reverse phase HPLC assay has been reported to analyze TX-100 in cell lysate samples with a determined LOD level of 0.0003% (3 ppm) [10]. In addition, one World Health Organization (WHO) technical report sets the permitted residual level of TX-100 in human blood products as ≤25 ppm from a safety perspective [11]. Finally, MTX has been used for the treatment of different diseases including cancer, leukemia, rheumatoid arthritis and psoriasis [12,13],

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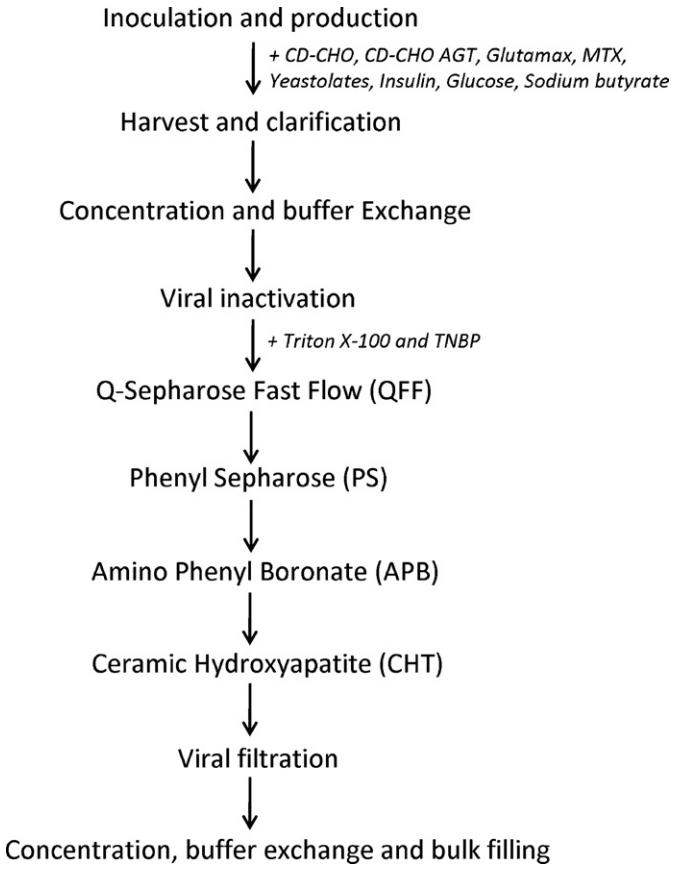

Fig. 1. Schematic showing rHuPH20 manufacturing processes. CD-CHO and CD-CHO AGT are protein-free, chemically defined media optimized for the growth of CHO cells and expression of recombinant proteins. Glutamax is a cell culture medium containing a dipeptide, L-alanyl-L-glutamine to prevent degradation and ammonia build-up. The Q-Sepharose Fast Flow (QFF), Phenyl Sepharose (PS), Amino Phenyl Boronate (APB) and Ceramic Hydroxyapatite (CHT) represent four sequential column purification stages.

and various analytical methods such as capillary electrophoresis (CE) [14,15] and HPLC [16,17] have been developed to determine and quantitate MTX in human plasma and urine samples down to a ppb level. However, very limited information is available about analytical monitoring and quantitation of yeastolates, TX-100 or MTX from a process development standpoint. Concomitantly, there has been increasing interest from regulatory agencies about process-related impurities and how the industry ensures that these components are removed during protein purification processes. Therefore, development of validated analytical methods that can accurately, precisely, and reliably measure these process-related impurities would be extremely useful for regulatory guidance and future process development studies.

The aim of this study was to develop a liquid chromatography method that can simultaneously analyze three process-related impurities: yeastolates, TX-100 and MTX. The method utilizes a Jupiter C4 column coupled with a UV detection monitored at multiple wavelengths: yeastolates at 257 nm, TX-100 at 280 nm and MTX at 302 nm, respectively. The method was validated in terms of selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy. As a specific example, the rHuPH20 in-process manufacturing materials from viral inactivation, QFF filtered, PS filtered, APB filtered, CHT filtered and final API were tested using the developed method. Varying levels of yeastolates, TX-100 and MTX in these samples were accurately determined.

2. Experimental

2.1. Reagents and materials

HPLC grade water and acetonitrile (ACN) were purchased from Honeywell (Morristown, NJ, USA). HPLC grade trifluoroacetic acid (TFA) was purchased from EMD Chemicals (Gibbstown, NJ, USA). Gibco yeastolate ultrafiltrate (200 mg/mL solution) was purchased from Invitrogen (Carlsbad, CA, USA). Difco yeastolate ultrafiltrate (powder) was purchased from BD Biosciences (San Jose, CA, USA). Ultrafiltered yeast hydrolysate (powder) was purchased from Irvine Scientific (Santa Ana, CA, USA). Methotrexate was purchased from MP Biomedicals (Solon, OH, USA). Triton X-100 was purchased from Sigma–Aldrich (St Louis, MO, USA). The rHuPH20 in-process and final API manufacturing materials (lot 104D-250-05.1) were obtained from the manufacturing department of Halozyme and include viral inactivation, QFF filtered, PS filtered, APB filtered, CHT filtered, viral filtrate and final API samples.

2.2. Instrumentation

The HPLC system was an Agilent 1200 series with a binary pump, vacuum degasser, autosampler and column heater. A Jupiter C4 column (4.6 mm × 250 mm, 5 μm, Phenomenex) was used with gradient elution from mobile phase A (MPA, 0.05% TFA in water) to mobile phase B (MPB, 0.05% TFA in ACN) at a flow rate of 1.0 mL/min. The autosampler was maintained at 5 °C and the column temperature was set at 40 °C. The injection volume was 10 μL for all injections and the detection was monitored at 257 nm, 280 nm and 302 nm simultaneously using a diode array detector (DAD). The gradient conditions are listed below.

Time (min)	MPA (%)	MPB (%)
0.0	95	5
2.0	95	5
12.0	55	45
18.0	25	75
25.0	5	95
30.0	5	95
30.1	95	5
35.0	95	5

2.3. Sample preparation

2.3.1. Solution preparation for selectivity test

All yeastolate powders were first dissolved in water to prepare 200 mg/mL solutions. 35 μL of individual yeastolate lots (200 mg/mL) was added to 965 μL of mobile phase A to prepare a 7 mg/mL yeastolate stock solution. 10 μL of TX-100 (100%) was added to 990 μL of mobile phase A to prepare a 1% TX-100 stock solution. 20 μL of MTX (1 mM, formulated in 0.1 M NaOH) was added to 980 μL of mobile phase A to prepare a 0.02 mM MTX stock solution. Next, each of the above stock solutions was diluted 16 times by adding 62.5 μL of each stock solution to 937.5 μL of mobile phase A. The final concentration was 0.4375 mg/mL yeastolates, 0.0625% TX-100 or 1.25 μM MTX in the individual solutions.

LOD level yeastolates, TX-100 and MTX were spiked in three analyte-free, in-process materials to examine the selectivity of the method towards real sample matrices. Specifically, 225.2 μL of yeastolates (200 mg/mL, lot 651942), 17.5 μL of TX-100 (100%) and 100 μL of MTX (1 mM) were mixed with 4657.3 μL of mobile phase A. This represents a "yeastolates + TX-100 + MTX" combined stock solution with 9 mg/mL of yeastolates, 0.35% of TX-100 and 20 μM of MTX. Next, 20 μL of the above stock solution was added to 980 μL of mobile phase A to prepare an intermediate "yeastolates + TX-100 + MTX" combined solution with 0.18 mg/mL of yeastolates, 0.007% of TX-100 and 0.4 μM of MTX. Finally, 5 μL of the

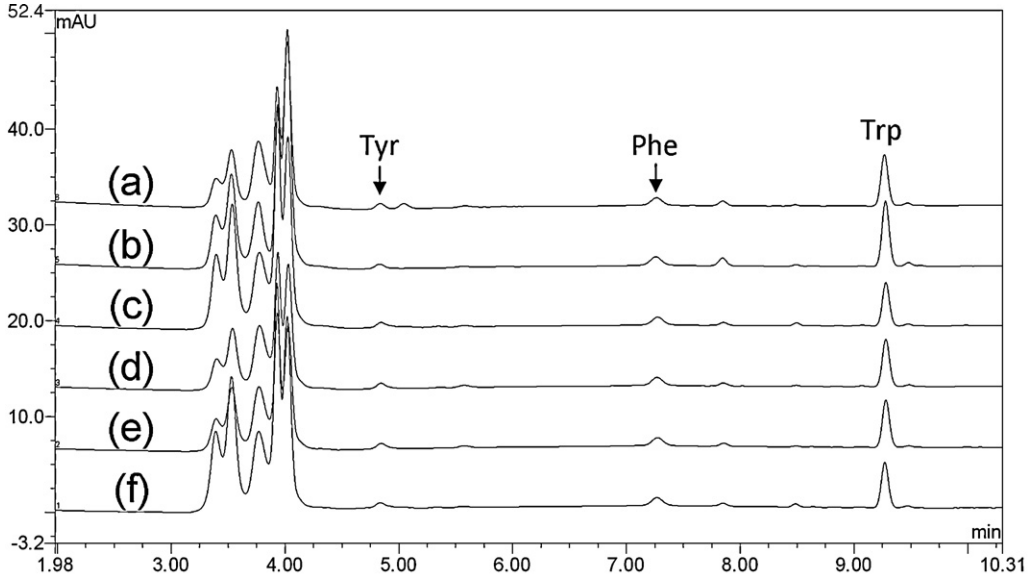

Fig. 2. Comparison of six yeastolate lots from three commercial suppliers: (a) Irvine yeastolate lot 9686381004, (b) Difco yeastolate lot 0209866, (c) Gibco yeastolate lot 438173, (d) Gibco yeastolate lot 1309797, (e) Gibco yeastolate lot 1323386 and (f) Gibco yeastolate lot 651942. Three peaks were identified to be tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp), respectively. The tryptophan peak was integrated for yeastolate quantitation.

intermediate solution was added to 95 μL of mobile phase A or APB-filtered, CHT-filtered and final API materials. The final concentrations of spiked PRI are 0.009 mg/mL yeastolates, 0.00035% TX-100, 0.02 μM MTX, corresponding to an LOD level of these impurities.

2.3.2. Solution preparation for linearity test

A "yeastolates + TX-100 + MTX" combined stock solution was prepared by adding 35 μL of yeastolates (200 mg/mL, lot 651942), 20 μL of MTX (1 mM) and 10 μL of TX-100 (100%) to 935 μL of mobile phase A. The concentrations of yeastolates, TX-100 and MTX are 7 mg/mL, 1% and 0.02 mM, respectively and represent the highest concentration point for the PRI calibration curve. The combined stock solution was serially diluted 4-fold each time with mobile phase A to prepare a series of "yeastolates + TX-100 + MTX" combined linearity solutions with dilution levels from 1:1 to 1:1024. These linearity solutions cover yeastolate concentrations from 0.007 to 7 mg/mL, TX-100 concentrations from 0.001% to 1% and MTX concentrations from 0.02 to 20 μM. A separate TX-100 calibration curve was created and used only for quantitation of TX-100 in "viral inactivation" material. Specifically, 20 μL of TX-100 (100%) was added to 980 μL of mobile phase A to prepare a stock solution of 2% TX-100. The stock solution was serially diluted 4-fold each time with mobile phase A to prepare a series of TX-100 linearity solutions by covering TX-100 concentrations from 0.0005% to 2%.

2.3.3. Solution preparation for accuracy test

Three different levels of "yeastolates + TX-100 + MTX" combined solutions were spiked to both mobile phase A and rHuPH20 final API material to examine the accuracy of the method. For the 1:4 dilution level, 10 μL of the "yeastolates + TX-100 + MTX" combined stock solution (1:1) was mixed with 30 μL of mobile phase A or rHuPH20 final API solution. For the 1:16 dilution level, 5 μL of the "yeastolates + TX-100 + MTX" combined stock solution (1:1) was mixed with 75 μL of mobile phase A or rHuPH20 final API solution. For the 1:64 dilution level, 5 μL of "yeastolates + TX-100 + MTX" combined solution (1:4) was mixed with 75 μL of mobile phase A or rHuPH20 final API solution. The accuracy of the method was also investigated by studying yeastolates, TX-100 and MTX at their LOQ levels. To determine LOQ levels, 135 μL of yeastolates (200 mg/mL, lot 651942), 10 μL of TX-100 (100%) and 90 μL of MTX (1 mM) were mixed with 765 μL of mobile phase A. This represents a "yeastolates + TX-100 + MTX" combined stock solution with 27 mg/mL of yeastolates, 1% of TX-100 and 90 μM of MTX. Next, an intermediate "yeastolates + TX-100 + MTX" combined solution was prepared by adding 20 μL of the above stock solution to 980 μL of mobile phase A. Finally, 5 μL of the intermediate solution was added to 95 μL of either mobile phase A or rHuPH20 manufacturing materials (viral inactivation, QFF, PS, APB, CHT filtered and final API). The final concentrations of spiked PRI are 0.027 mg/mL yeastolates, 0.001% TX-100, 0.09 μM MTX, corresponding to an LOQ level of yeastolates, TX-100 and MTX which will be addressed in later sections.

3. Results and discussion

3.1. Comparison of yeastolates from different manufacturers

Yeastolates are undefined mixtures of amino acids, peptides, carbohydrates, vitamins and minerals with other potential components [18]. To develop a method for yeastolate analysis, it is of primary importance to determine if the starting components of yeastolates are from lot to lot or from different manufacturers. We have compared six representative yeastolate lots from three commercial suppliers using the established HPLC method. All yeastolate lots were diluted to 0.4375 mg/mL and analyzed by RP-HPLC using identical method parameters. The results are depicted in Fig. 2. Due to the complexity in yeastolate composition, only three peaks have been identified to be tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Trp). Each yeastolate lot displays multiple peaks with the retention time ranging from 3 to 9 min. The individual peaks are fairly reproducible but their relative intensities are slightly varied. %CV of the six batches (calculated based on the tryptophan peak) is about 16%, which is reasonable considering the fact that they are from three different manufacturers. %CV of the four Gibco yeastolate lots from the same manufacturer (Fig. 2c–f) is only 4.8%. However, for a proper quantitation, it is advised that the yeastolate batch (or lot) used in calibration samples be identical to that used in actual manufacturing materials. In a separate stability study, a single yeastolate lot was used to prepare a solution at 0.4375 mg/mL at each time point ($n = 10$). %RSD of the yeastolate peak measured over a period of six months was only 2.3%, indicating that

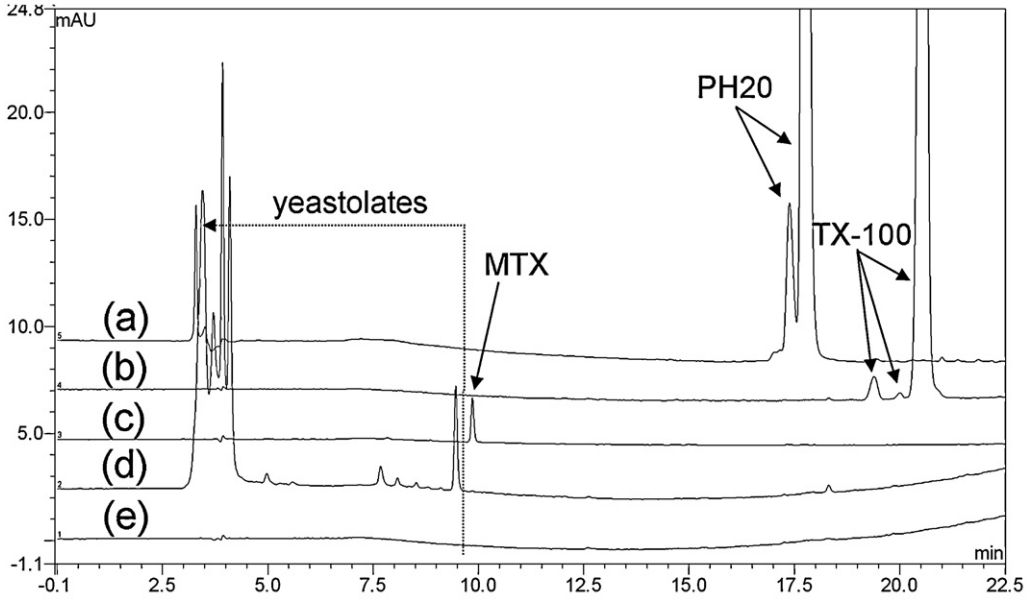

Fig. 3. Selectivity of the method evaluated by injecting (a) 1 mg/mL rHuPH20 API, (b) 0.0625% TX-100, (c) 1.25 μM MTX, (d) 0.4375 mg/mL yeastolates and (e) a water blank. The UV profiles shown were collected from different wavelengths with the water blank and yeastolates at 257 nm, TX-100 and rHuPH20 at 280 nm and MTX at 302 nm.

yeastolates are relatively stable (data not shown). Considerable effort has been devoted to establish an appropriate integration strategy for yeastolate quantitation. Our initial endeavor was to integrate all yeastolate peaks in Fig. 2. This approach was found to be problematic due to buffer and/or solvent front interference at the early elution time (data not shown). A satisfactory result was finally achieved by monitoring a single peak at one specific retention time (the tryptophan peak in Fig. 2) that was well-resolved from the rest of the peaks and consistent across tested batches. This strategy was used for all yeastolate quantitation throughout the study.

3.2. Selectivity

Selectivity describes the ability of the method to assess the desired analyte in the presence of other components. In this study the selectivity of the method was examined by injecting individual solutions of yeastolates, TX-100, MTX, rHuPH20 and a water blank. The results are summarized in Fig. 3. The UV profiles for each component were collected at variable wavelengths with yeastolates at 257 nm, TX-100 and rHuPH20 at 280 nm and MTX at 302 nm. Yeastolates still displayed multiple peaks ranging from 3 to 9 min. MTX was a single peak at 9.9 min. TX-100 displayed a major peak at 20.6 min accompanied by two small related peaks. rHuPH20 was the biopharmaceutical API comprising two main peaks that eluted around 17.5 min. All these components eluted at different retention times. This observation was further verified by injecting a combined solution of yeastolates, TX-100, MTX and rHuPH20 with the same method. A clear separation of these components was achieved at each wavelength and no cross-interference was observed (data not shown). Therefore, a combined solution of yeastolates, TX-100 and MTX was directly used for the quantitation studies of the individual components.

Due to the complexity of in-process materials, it is also necessary to examine the selectivity of the method towards real sample systems. Here three in-process materials (APB filtered, CHT filtered and final API), which were found analyte-free, were spiked with yeastolates, TX-100 and MTX at their LOD levels. The corresponding chromatograms are compared in Figs. 4–6 for yeastolates, TX-100 and MTX, respectively. Even at low LOD levels, no background signals from APB filtered, CHT filtered and final API interfered with the detection of yeastolates, TX-100 and MTX. The same solutions were also spiked with LOQ level yeastolates, TX-100 and MTX and no interference was observed (data not shown).

3.3. Linearity, LOD and LOQ

Linearity is an important component in method development and determines whether a method is suitable for analyte quantitation over a range of concentrations. In this study we quantitated yeastolates, TX-100 and MTX in a single method by coupling HPLC separation with multiple wavelength detection. A combined stock solution of yeastolates, TX-100 and MTX was prepared and serially diluted to cover concentrations of yeastolates from 0.014 to 7 mg/mL, TX-100 from 0.001% to 1% and MTX from 0.02 to 20 μM (Section 2.3.2). As mentioned previously, only the tryptophan peak from yeastolates was used for quantitation. TX-100 contained three peaks as in Fig. 3 and all of them were integrated for TX-100 quantitation. MTX is a single peak and was integrated for quantitation. The linearity curves for each component were generated by plotting peak areas as a function of concentration and the calibration parameters are summarized in Table 1. Excellent linearity was observed for yeastolates, TX-100 and MTX analyses at both high and low concentration ranges.

The LOD and LOQ for yeastolates, TX-100 and MTX analyses were determined using signal-to-noise (S/N) ratios based on a series of diluted "yeastolates + TX-100 + MTX" combined solutions.

Table 1
Parameters of linearity curves for yeastolates, TX-100 and MTX analyses.

PRI identity	Concentration range	*y*-Intercept	Slope	r^2
Yeastolates	0.014–7 mg/mL	0.0188	60.049	1
	0.014–0.1 mg/mL	−0.0405	58.923	0.9999
TX-100	0.001–1%	−4.5973	9535.8	1
	0.001–0.016%	−1.7333	8660.1	1
MTX	0.02–20 μM	0.7973	10,806	0.9996
	0.02–0.3 μM	0.0033	10,874	1

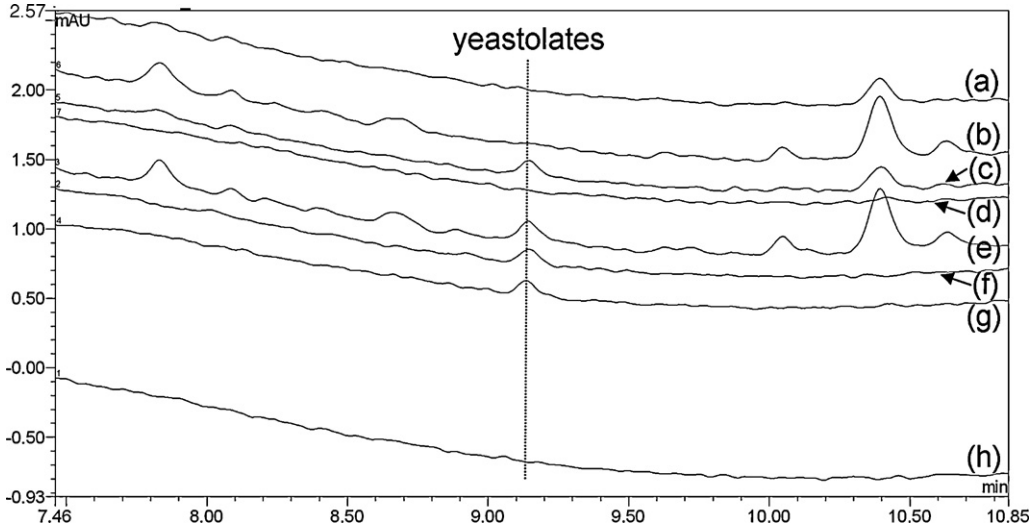

Fig. 4. Selectivity of the method evaluated by injecting (a) APB filtered, (b) final API, (c) LOD level yeastolates spiked in APB filtered, (d) CHT filtered, (e) LOD level yeastolates spiked in final API, (f) LOD level yeastolates spiked in MPA, (g) LOD level yeastolates spiked in CHT filtered and (h) a water blank. The UV chromatograms were collected at 257 nm.

Table 2
Summary of LOD and LOQ for yeastolates, TX-100 and MTX analyses.

PRI identity	LOD	LOQ
Yeastolates	0.009 mg/mL (9 ppm)	0.027 mg/mL (27 ppm)
TX-100	0.00035% (3.5 ppm)	0.001% (10 ppm)
MTX	0.02 μM (9 ppb)	0.09 μM (40.5 ppb)

The LOD and LOQ corresponded to signal-to-noise (S/N) ratios of approximately 3 and 10, respectively. The results are summarized in Table 2. The LOD and LOQ for yeastolates, TX-100 and MTX were further investigated using real matrix systems. Here LOD level yeastolates, TX-100 and MTX were spiked in three analyte-free, in-process materials. As clearly demonstrated in Figs. 4–6, similar S/N ratios were obtained corresponding to their LOD levels. The LOQ was also confirmed using same spiked solutions (data not shown). Therefore, both LOD and LOQ obtained in Table 2 are applicable to both academic solutions and more complicated in-process rHuPH20 materials.

3.4. Precision and accuracy

The precision of the method for yeastolates, TX-100 and MTX analyses was determined using six replicate injections of one "yeastolates + TX-100 + MTX" combined solution at their LOQ levels containing 0.027 mg/mL yeastolates, 0.001% TX-100 and 0.09 μM MTX. The %RSD for yeastolates, TX-100 and MTX analyses was 1.4%, 1.6% and 3.0%, respectively.

The accuracy of the method was evaluated using spiked samples at different PRI concentration levels (at dilution factors of 1:4, 1:16 and 1:64). Specific amounts of "yeastolates + TX-100 + MTX" combined solution were spiked in both mobile phase A and rHuPH20 final API solution (Section 2.3.3). The spiked solutions were injected and their peak areas were directly compared. The results are summarized in Table 3. The percent recovery for each component was calculated using the following formula:

$$\%\text{Recovery} = \frac{\text{Area in final API}}{\text{Area in MPA}} \times 100$$

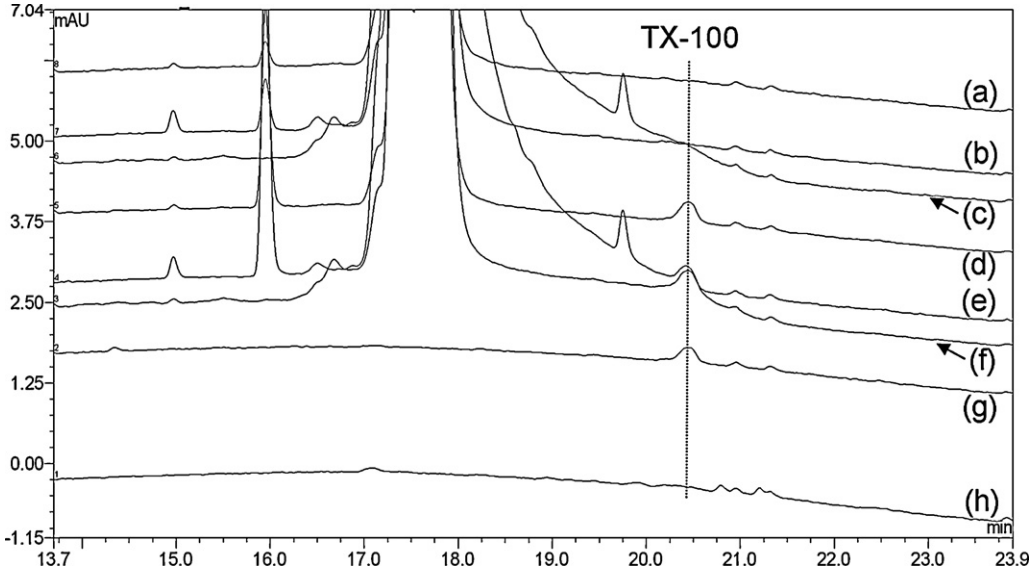

Fig. 5. Selectivity of the method evaluated by injecting (a) APB filtered, (b) CHT filtered, (c) final API, (d) LOD level TX-100 spiked in APB filtered, (e) LOD level TX-100 spiked in CHT filtered, (f) LOD level TX-100 spiked in final API, (g) LOD level TX-100 spiked in MPA and (h) a water blank. The UV chromatograms were collected at 280 nm.

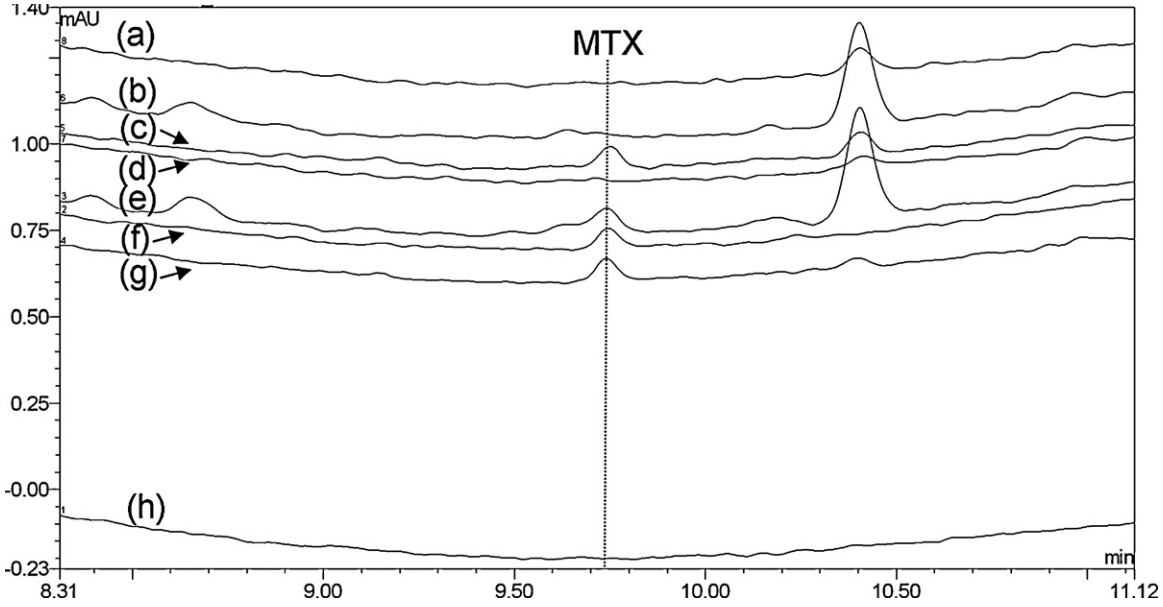

Fig. 6. Selectivity of the method evaluated by injecting (a) APB filtered, (b) final API, (c) LOD level MTX spiked in APB filtered, (d) CHT filtered, (e) LOD level MTX spiked in final API, (f) LOD level MTX spiked in MPA, (g) LOD level MTX spiked in CHT filtered and (h) a water blank. The UV chromatograms were collected at 302 nm.

The percent recovery for yeastolates, TX-100 and MTX was 98–105%, 93–100% and 97–105%, respectively.

The accuracy of the method was further investigated at lower PRI concentrations. Specific amounts of "yeastolates + TX-100 + MTX" combined solutions corresponding to their LOQ levels were spiked in six rHuPH20 in-process and final API materials (viral inactivation, QFF, PS, APB, CHT filtered and final API from lot 104D-250-05.1) (Section 2.3.3). The same levels of "yeastolates + TX-100 + MTX" combined solutions were also spiked in mobile phase A and used as nominal concentrations for accuracy measurements. Note that for all rHuPH20 in-process and final API materials, certain levels of yeastolates, TX-100 or MTX may be pre-existing. Therefore, the percent recovery for each component can be calculated using the following formula:

$$\%\text{Recovery} = \frac{\text{Area in spiked sample} - \text{Area in sample alone} \times 0.95}{\text{Area in MPA}} \times 100$$

where 0.95 is the dilution factor in accuracy sample preparation (Section 2.3.3). The results are summarized in Table 4. The percent recoveries for yeastolates, TX-100 and MTX were 94–108%, 66–106% (except the value from viral inactivation material) and 94–110%, respectively. Low percent recovery of TX-100 was found for viral inactivation, QFF and PS filtered materials. This is not surprising since the viral inactivation material has the highest TX-100 level (~1%) and QFF and PS represent the first two column purification steps during the manufacture processing of rHuPH20. Interference from other components such as protein by-products that eluted at similar retention time as TX-100 caused the poor recovery for these materials (data not shown). Fortunately these components were eliminated in the following column purification steps and posed no interference to TX-100 quantitation in the later stages.

It should be noted that the current established method has been successfully transferred to CMOs and validated in a GMP environment. Intermediate precision was performed by varying the following parameters: analysts ($N = 2$), columns, instruments, and time. The robustness of the method was examined by varying the following parameters: (1) mobile phases was prepared with 0.04% or 0.06% TFA (vs 0.05% TFA), (2) flow rate was changed to 0.9 or 1.1 mL/min (vs 1.0 mL/min), (3) column temperature was changed to 35 or 45 °C (vs 40 °C) and (4) autosampler temperature was changed to 25 °C (vs 5 °C). All results obtained met the acceptance criteria (data not shown), indicating that the method is suitable for determination and quantitation of yeastolates, TX-100 and MTX.

3.5. Determination of yeastolates, MTX and TX-100 in rHuPH20 in-process and final API samples

Having established the feasibility and suitability of the method, we proceeded to the measurement of yeastolates, TX-100 and MTX in rHuPH20 manufacturing materials. The samples were collected from different purification stages including viral inactivation, QFF, PS, APB, CHT and final API. The samples were injected without any dilution and the result was compared for yeastolates, TX-100 and MTX at different wavelengths. A "yeastolates + TX-100 + MTX" combined solution at a dilution level of 1:16 was injected as a reference. Fig. 7 summarizes the comparison of yeastolate peaks from different samples at 257 nm. Peaks at analytes' retention time were further confirmed by comparing their UV spectra (by using diode

Table 3
Summary of accuracy results by spiking "yeastolates + TX-100 + MTX" combined solutions at different concentration levels.

Dilution level	Spiked condition	Yeastolates (mAU s)	%Recovery	TX-100 (mAU s)	%Recovery	MTX (mAU s)	%Recovery
1:64	In MPA	6.7		166.7		3.5	
	In final API	6.6	99	155.0	93	3.4	97
1:16	In MPA	26.9		696.6		13.7	
	In final API	28.3	105	695.1	100	14.4	105
1:4	In MPA	87.1		2196.2		44.0	
	In final API	85.1	98	2135.9	97	43.3	98

Table 4
Summary of accuracy results by spiking "yeastolates + TX-100 + MTX" combined solutions at LOQ levels.

Samples	Spiked condition	Yeastolates (mAU s)	%Recovery	TX-100 (mAU s)	%Recovery	MTX (mAU s)	%Recovery
	In MPA	1.4		9.5		1.12	
Viral inactivation	SPL alone	10.2		11,667.7		10.96	
	Spiked	11.1	107	11,054.6	−313	11.51	98
	In MPA	1.5		10.6		0.95	
QFF filtered	SPL alone	3.0		6.9		0.22	
	Spiked	4.4	103	15.3	83	1.10	94
	In MPA	1.6		9.8		1.00	
PS filtered	SPL alone	0.4		2.9		0.00	
	Spiked	2.1	108	9.2	66	1.10	110
	In MPA	1.7		9.5		0.96	
APB filtered	SPL alone	0.0		0.0		0.00	
	Spiked	1.6	94	9.0	95	1.00	104
	In MPA	1.6		9.3		0.96	
CHT filtered	SPL alone	0.0		0.3		0.00	
	Spiked	1.5	94	8.7	91	1.00	104
	In MPA	1.6		9.4		1.00	
Final API	SPL alone	0.0		0.0		0.00	
	Spiked	1.6	100	10.0	106	1.10	110

Note: SPL is the abbreviation of "sample".

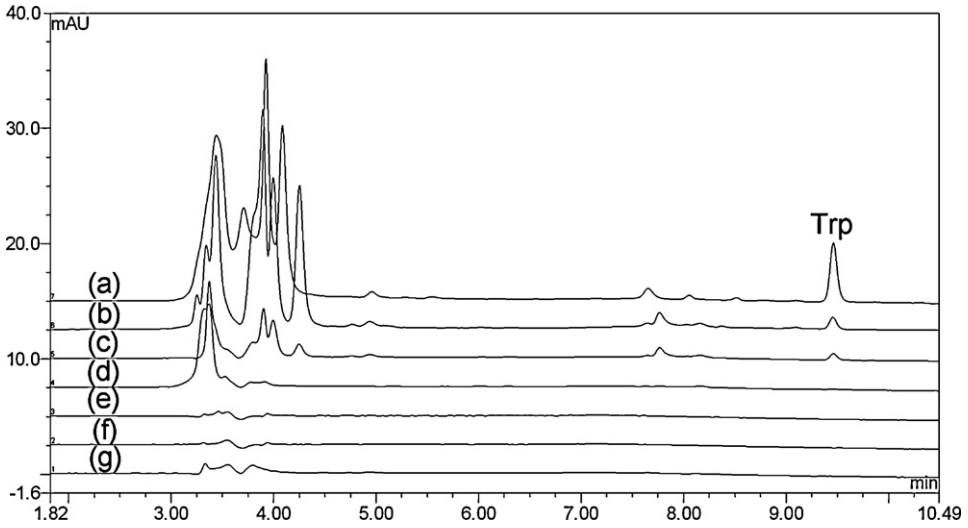

Fig. 7. Comparison of (a) a "yeastolates + TX-100 + MTX" combined standard solution (1:16) and rHuPH20 in-process samples of (b) viral inactivation, (c) QFF filtered, (d) PS filtered, (e) APB filtered, (f) CHT filtered and (g) final API materials. The UV profiles were collected at 257 nm and the tryptophan peak was used for yeastolate quantitation.

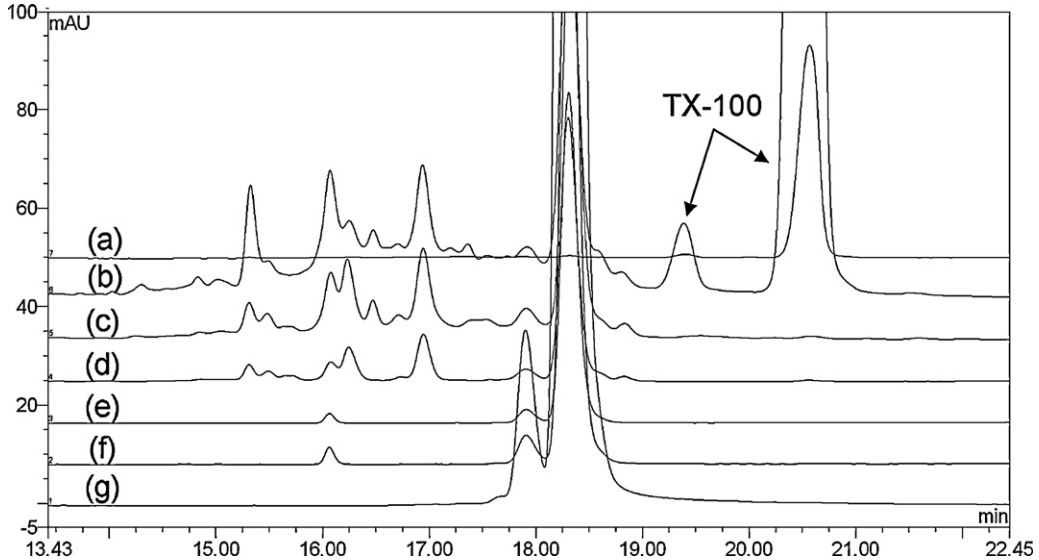

Fig. 8. Comparison of (a) "yeastolates + TX-100 + MTX" combined standard solution (1:16) and rHuPH20 in-process samples of (b) viral inactivation, (c) QFF filtered, (d) PS filtered, (e) APB filtered, (f) CHT filtered and (g) final API. The UV profiles shown were collected at 280 nm.

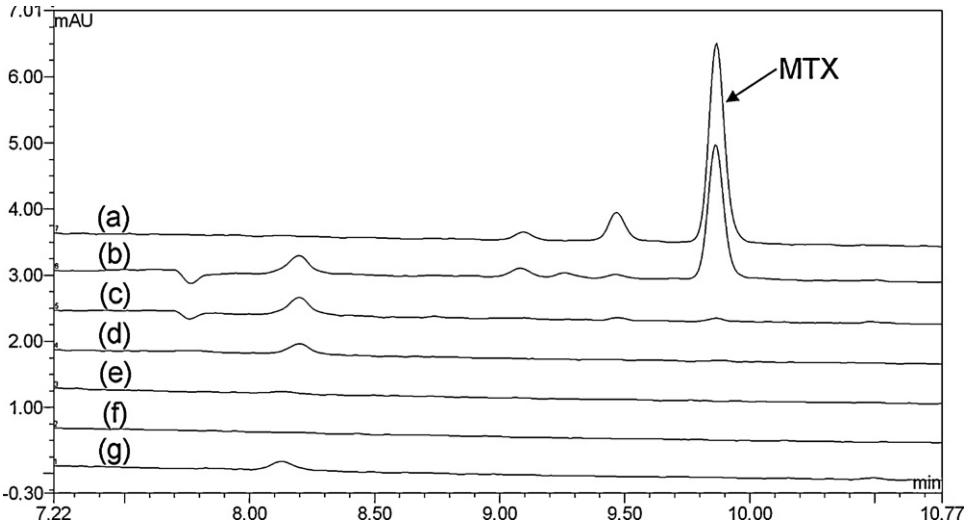

Fig. 9. Comparison of (a) "yeastolates + TX-100 + MTX" combined standard solution (1:16) and rHuPH20 in-process samples of (b) viral inactivation, (c) QFF filtered, (d) PS filtered, (e) APB filtered, (f) CHT filtered and (g) final API. The UV profiles shown were collected at 302 nm.

Table 5
Determination of yeastolates, TX-100 and MTX in rHuPH20 manufacturing materials.

Samples	Yeastolates (mg/mL)	TX-100 (%)	MTX (μM)
Viral inactivation	0.09	1.2266[a]	0.75
QFF filtered	0.046	0.0014	<LOD
PS filtered	<LOD	0.0010	<LOD
APB filtered	<LOD	<LOD	<LOD
CHT filtered	<LOD	<LOD	<LOD
Final viral filtrate	<LOD	<LOD	<LOD
Final API	<LOD	<LOD	<LOD

[a] The amount of TX-100 in viral inactivation material was found out of the calibration range listed in Table 1. A new calibration curve was created covering TX-100 concentrations from 0.0005% to 2% ($y = 9553x + 19.018$, $r^2 = 1$) and used for its quantitation. See Section 2.3.2 for corresponding solution preparation.

array detector) with analyte peaks in the reference solution. The results clearly show that after the first two column purification steps (QFF and PS), yeastolates were completely eliminated. Fig. 8 shows the comparison of TX-100 peaks at 280 nm. A high level of TX-100 (~1% in Table 5) was observed in rHuPH20 viral inactivation sample but was removed after the first QFF column purification step. The comparison of MTX at 302 nm is demonstrated in Fig. 9. The viral inactivation sample also has the highest MTX level at about 0.75 μM (Table 5) and was eliminated after the QFF column purification step. The levels of yeastolates, TX-100 and MTX in all rHuPH20 samples were determined by direct comparison with their corresponding calibration curves (Table 1) and the results are summarized in Table 5.

4. Conclusion

We present for the first time the development and validation of an RP-HPLC method for the simultaneous determination of three process-related impurities (yeastolates, TX-100 and MTX) that are commonly used in cell-based manufacturing processes. During process qualification it is necessary to demonstrate impurity removal during each purification step. This method allows the determination of three impurity levels in one assay using basic lab setups which is a significant improvement with respect to both time and resource constraints. The LOQ achieved for each impurity is appropriate for a state-of-the-art manufacturing process. The method was validated with respect to selectivity, linearity, LOD, LOQ, precision and accuracy studies. The method was successfully applied to the quantitative analyses of yeastolates, TX-100 and MTX in most rHuPH20 in-process and final API manufacturing materials and trace levels of these impurities were accurately measured. Due to strong matrix effect, the amount of TX-100 in viral inactivation, QFF and PS filtered materials was not accurately determined.

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