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Determination of Impurities in New Promising Antioxidants Bis-[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propyl]sulfide and Dodecyl(3,5-dimethyl-4-hydroxybenzyl)sulfide

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Abstract

Introduction. Dodecyl(3,5-dimethyl-4-hydroxybenzyl)sulfide (T1) and bis-[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propyl]sulfide (T2) are the collaborative development of Novosibirsk State Pedagogical University and Novosibirsk Research Institute of Antioxidants. It was revealed in several experiments and research works that these substances have antioxidant, anti-inflammatory, hepatoprotective, cytoprotective, haemorheological activities. These facts make the objects of study promising medicinal antioxidant drugs. Consequently it's necessary for the future production quality control to have standards and analytical methods for substances analysis.

Aim. Impurities methods development and validation for the new biologically active substances T1 and T2.

Materials and methods. HPLC method with UV-detection on 278 nm was carried out for the determination of impurities in objects of study. HPLC analysis were performed on ZORBAX SB-C18 (5 μm, 150 × 4,6 mm) column with isocratic regimen and with use of the acetonitrile:water mixture (T1) or acetonitrile (T2) as a mobile phase.

Results and discussion. It was find out, that T1 has two unidentified impurities with concentration not more than 0,1 % during the shelf life. The chromatogramm of T2 has a peak of by-product of synthesis T2 – bis-[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propyl]disulfide (T2-DS). Biologically safety of T2-DS was demonstrated in the previous works and the concentration of T2-DS was established to be not more, than 2,5 %. Furthermore, there was the one more unidentified impurity with concentration less, than 0,1 % on the chromatogram of T2. The developed HPLC methods were validated on characteristics «specificity», «linearity», «precision», «limit of quantification», «accuracy», «range».

Conclusion. Methods for the determination of impurities in T1 and T2 were validated on the listed parameters. All the results meet the acceptance criteria: peaks on the chromatogramms are clearly separated; the correlation coefficients (*r*) are not more, than 0,980; accuracy was proved by linearity parameters; the value of the relative standard deviation is less, than 5 %; the intermediate precision for the both methods was proved by Fisher's criterion and Student's t-test.

Keywords: impurities, dodecyl(3,5-dimethyl-4-hydroxybenzyl)sulfide, bis-[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propyl]sulfide, HPLC, validation, standardization

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

Contribution of the authors. Tatiana G. Shinko, Semen Ye. Yagunov, Natalya V. Kandalintseva, Olga I. Prosenko and Pavel I. Pinko designed the experiment. Tatiana G. Shinko, Svetlana V. Terentyeva and Yelena A. Ivanovskaya – performed methods validation. All authors participated in the discussion of the article and contributed to the final text of the article.

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Определение примесей в перспективных антиоксидантах бис-[3-(3,5-ди-трет-бутил-4-гидроксифенил)пропил]сульфиде и додецил(3,5-диметил-4-гидроксибензил)сульфиде

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Резюме

Введение. Додецил(3,5-диметил-4-гидроксибензил)сульфид (Т1) и бис-[3-(3,5-ди-трет-бутил-4-гидроксифенил)пропил]сульфид (Т2) – совместная разработка кафедры химии Новосибирского государственного педагогического университета и Новосибирского института антиоксидантов. В исследованиях и экспериментах была показана антиоксидантная, противовоспалительная, гепатопротекторная, цитопротекторная, гемореологическая активность данных веществ, что позволяет считать их перспективными лекарственными антиоксидантами. Для контроля качества при последующем производстве субстанций указанных соединений необходимо разработать параметры стандартизации и составить Проект Нормативной документации.

Цель. Разработка и валидация методик определения примесей новых перспективных биологически активных веществ Т1 и Т2.

Материалы и методы. Определение примесей проводили методом ВЭЖХ с УФ-детектированием при длине волны 278 нм на колонке ZORBAX SB-C18 (5 мкм, 150 × 4,6 мм) в изократическом режиме с использованием в качестве подвижной фазы смеси ацетонитрил – вода 95 : 5 при анализе Т1 и ацетонитрила при анализе Т2.

Результаты и обсуждение. В ходе разработки методик было установлено присутствие в исследуемых образцах Т1 двух неидентифицированных примесей, содержание которых в течение срока годности не превышает 0,1 %. На хроматограмме Т2 обнаружены побочный продукт синтеза – бис-[3-(3,5-ди-трет-бутил-4-гидроксифенил)пропил]дисульфид (Т2-ДС) с установленной биологической безопасностью и пределом содержания 2,5 %, а также неидентифицированная примесь с содержанием не более 0,1 %. Проведена валидация разработанных методик ВЭЖХ по характеристикам «Специфичность», «Предел количественного определения», «Линейность», «Прецизионность», «Правильность», «Аналитическая область».

Заключение. Методики определения примесей Т1 и Т2 валидированы по указанным выше параметрам. Полученные результаты удовлетворяют критериям приемлемости: вещества и примеси четко разделяются на хроматограммах между собой; коэффициент корреляции (*r*) при определении линейности для примесей превышает 0,980; свободный член уравнений линейной зависимости статистически значимо не отличается от нуля; величина относительного стандартного отклонения (RSD%) не более 5 %; рассчитанные значения критериев Стьюдента и Фишера не превышают табличные. Разработанные методики могут применяться для контроля качества опытных образцов и в будущем при производстве субстанций исследуемых веществ.

Ключевые слова: родственные примеси, додецил(3,5-диметил-4-гидроксибензил)сульфид, бис-[3-(3,5-ди-трет-бутил-4-гидроксифенил) пропил]сульфид, ВЭЖХ, валидация, стандартизация

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. Т. Г. Шинко, С. Е. Ягунов, Н. В. Кандалинцева, О. И. Просенко, П. И. Пинко разработали эксперимент. Т. Г. Шинко, С. В. Терентьева, Е. А. Ивановская провели валидацию методик. Все авторы участвовали в обсуждении статьи, внесли вклад в окончательную рукопись.

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INTRODUCTION

Dodecyl(3,5-dimethyl-4-hydroxybenzyl)sulfide (T1) and bis-[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propyl]sulfide (T2) (Figure 1) are polyfunctional phenolic sulfur-containing antioxidants. These molecules were synthesized by common work of Novosibirsk State Pedagogical University and Novosibirsk Research Institute of Antioxidants.

The biological activity researches revealed that these substances possess antioxidant, anti-inflammatory, hepatoprotective and cytoprotective activities [1–4]. It was also found out that both substances are able to increase the anti-tumor effect of cytostatic drugs [5, 6]. In addition T1 was established to have haemoreological, antiplatelet and antiaggregation effects. These properties provide the protective effect in cases of cerebral ischemia [3, 7]. The efficacy and the safety of T1 and

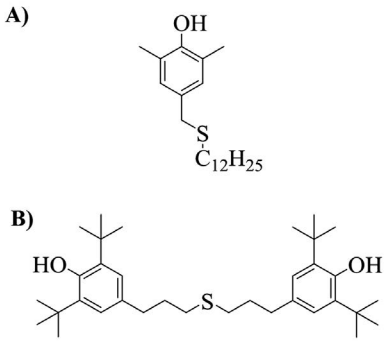

Figure 1. Chemical structure of dodecyl(3,5-dimethyl-4-hydroxybenzyl)sulfide-T1 (A) and bis-[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propyl]sulfide-T2 (B)

T2 were also investigated. It was revealed that these substances have no toxic effect and don't exhibit any mutagenic and genotoxic properties [8, 9]. With all these facts T1 and T2 can be considered as the promising

antioxidant drugs for complex therapy of such diseases as hepatitis, cancer, atherosclerosis, ischemia.

The development of specifications and normative documentation projects for T1 and T2 is necessary in the case of future production and registration of these substances as medicines. One of the quality parameters for medicines is "Impurities". Therefore **the aim** of this study is to develop and validate the analytical methods for determination of impurities in T1 and T2.

MATERIALS AND METHODS

Study objects. The methods development and validation were performed on samples of dodecyl(3,5-dimethyl-4-hydroxybenzyl)sulfide (series 2020-11-28) and bis-[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propyl]sulfide (series D-501), provided by Novosibirsk Research Institute of Antioxidants.

Reagents. The following reagents were used for the study: acetonitrile (Sigma-Aldrich, USA, for HPLC >99,9 %), ethanol (GOST R 51652-200).

Equipment. Determination of impurities was performed on high performance liquid chromatography system Agilent 1220 Infinity LC with UV-detector.

Sample and reference solutions of T1. Sample solution: About 0,500 g (an accurate weight) of T1 was dissolved in ethanol and diluted to 100,0 ml with the same solvent (5 mg/ml). Reference solution: 1,0 ml of the sample solution was diluted to 10,0 ml with ethanol. After mixing 1,0 ml of this solution was diluted to 100,0 ml with the same solvent (5 µg/ml).

System suitability test solution (T1): About 0,050 g (an accurate weight) of hydroquinone was transferred to a 200 ml volumetric flask. Then about 100 ml of ethanol and 1,0 ml of sample solution of T1 were added and mixed for dissolution. Then solution in the flask was made up to the mark with ethanol and mixed.

Sample and reference solutions of T2. Sample solution: About 0,500 g (an accurate weight) of T2 was dissolved in 5 ml of acetonitrile and diluted to 25,0 ml with the same solvent. After mixing, to 400,0 µl of this solution 600,0 µl of acetonitrile were added (8 mg/ml). Reference solution A: 1,0 ml of the sample solution was diluted to 10,0 ml with acetonitrile. After mixing, 1,0 ml of this solution was diluted to 100,0 ml with the same solvent (8 µg/ml). Reference solution B: About 0,050 g (an accurate weight) of bis-[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propyl]disulfide (T2-DS) was dissolved in 5 ml of acetonitrile and diluted to 25,0 ml with the same solvent. After mixing to 100,0 µl of this solution 900,0 µl of acetonitrile were added (0,2 mg/ml).

Selected chromatographic conditions are presented in Table 1.

Table 1. HPLC Conditions for determination of relative substances in T1 and T2

Parameter	Value	
	T1	T2
Mobile phase	Acetonitrile : water 95 : 5	Acetonitrile
Column	ZORBAX SB-C18 (5 µm, 150 × 4,6 mm)	ZORBAX SB-C18 (5 µm, 150 × 4,6 mm)
Column temperature	40 °C	40 °C
Flow rate	2 ml/min	2 ml/min
Detector	UV, 278 nm	UV, 278 nm
Probe	10 µl	5 µl

RESULTS AND DISCUSSION

Methods development. HPLC conditions for determination of related substances in the objects of study were selected during the synthesis process development for T1 and T2, and were used for the purity control of the pilot samples. The maximum of UV-absorption (278 nm) was chosen for detection, because this value is also nearer the maximum of UV-absorption of some initial synthesis substances and by-products. According to the technological scheme of synthesis of T2, the main by-product (T2-DS) content is not less than 2 %. Therefore the safety and toxicity of T2-DS were studied. As the result of safety establishing the upper limit of T2-DS content was determined to be not more than 2,5 %. The content of other impurities determined on chromatograms was not more than 0,1 %. In such a case these impurities can be determined as unspecified (according to General Pharmacopeial monograph 1.1.0006.15 "Pharmaceutical substances" of the State Pharmacopoeia of Russian Federation, edition XIV the upper limit for unspecified impurities is not more than 0,1 % and the disregard limit is 0,05 %).

Validation of HPLC methods for determination of T1 and T2 impurities. Methods validation was based on General Pharmacopeial monograph 1.1.0012.15 "Analytical methods validation" of the State Pharmacopoeia of Russian Federation, edition XIV. Methods were validated by parameters "specificity", "linearity", "limit of quantitation", "precision", "accuracy", "range" [10–14].

"Specicificity" of the methods was confirmed by the absence of peaks with retention time of T1, T2, T2-DS and other impurities on the chromatograms of solvents, and also by resolution ($R_s \geq 2$) between every two peaks on sample solutions chromatograms.

According to the results, there are no peaks with retention time of T1, T2 and impurities on blank-cromatograms (Figure 2, 3). Resolutions between peaks on the chromatogram of sample solution of T1 are $R_{S1} = 6,35$ and $R_{S2} = 10,17$. Resolutions between peaks on the chromatogram of sample solution of T2 are $R_{S1} = 2,74$ and $R_{S2} = 4,20$. These values meet the acceptance criteria.

Chromatographic system suitability for T1 was proven if the following conditions on the chromatogram of the system suitability test solution were met: resolution (R_s) between peaks of hydroquinone and T1 was not less, than 10; chromatographic column efficacy (N) for peak of T1 wass not less than 5000 theoretical plates; T1 peak symmetry factor (A_s) wass not less than 0,9 and not more than 1,5; relative standard deviation (RSD,%) of T1 peak area wass not more than 2 %.

Chromatographic system suitability for T2 was proven if the following conditions on the chromatogram of sample solution of T2 were met: resolutions (R_s) between peaks on the chromatogram were not less than 2; chromatographic column efficacy (N) for peak of T2 was not less than 5000 theoretical plates; T2 peak symmetry factor (A_s) was not less than 0,8 and not more than 1,5; relative standard deviations (RSD,%) of peaks areas were not more than 2 %. The results of system suitability test for T1 and T2 are given in Table 2.

"Linearity" of the methods was confirmed by linear dependence (linear regression coefficient $r \geq 0,980$) between the concentration data of T1 and T2 and peaks areas on the chromatograms of calibration solutions of T1 and T2. To perform the linearity test series of T1 and T2 calibration solutions with the concentration of impurities in the range of LOQ to 120–150 % of specification levels were made.

The results of linearity tests are shown in Figure 4 and Table 2. All the data meet the acceptance criteria ($r \geq 0,980$), methods are linear in the test concentration range.

Limits of quantitation for unspecified impurities, calculated by the value of signal standard deviation and angel coefficient of calibration plot (Table 2) are not more, than less calibration concentrations (disregard limits). Signal to noise ratios (S/N) on chromatograms of 0,05 % sample solutions of T1 and T2 (disregard limits) are 29,0752 and 34,829 respectively. These data meet the acceptance criteria (S/N ≥ 10).

"Accuracy" of methods was established by linearity tests results. It was accepted that there is no method systematic error if the free member (a) of linear dependence equation is not significantly different from zero. All the data meet the acceptance criteria (Table 2).

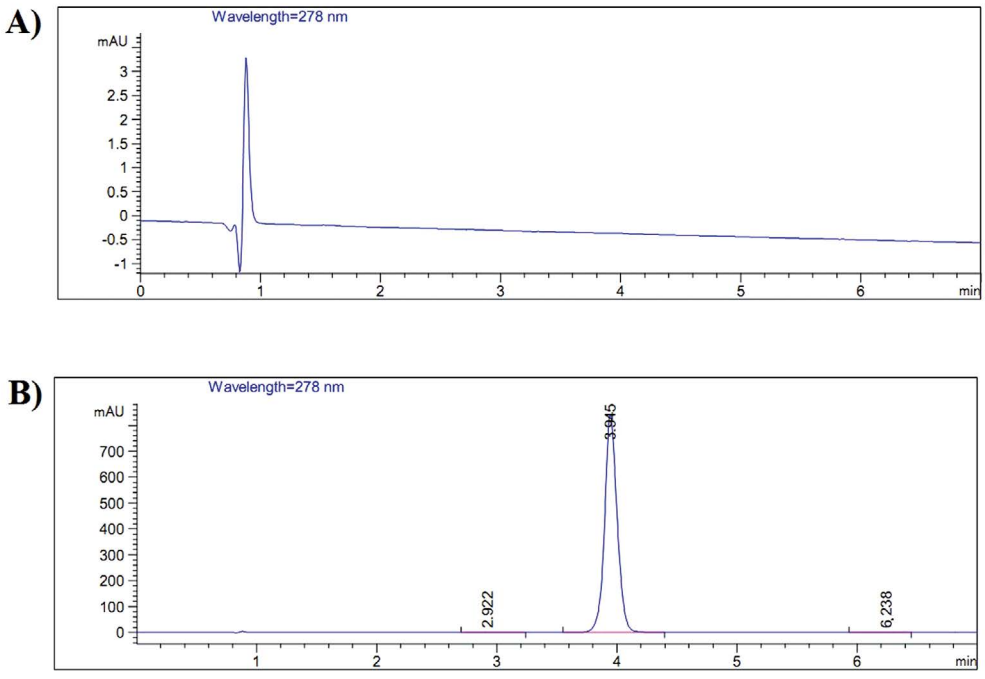

Figure 2. Chromatograms of a solvent (A) and test solution (B) of T1

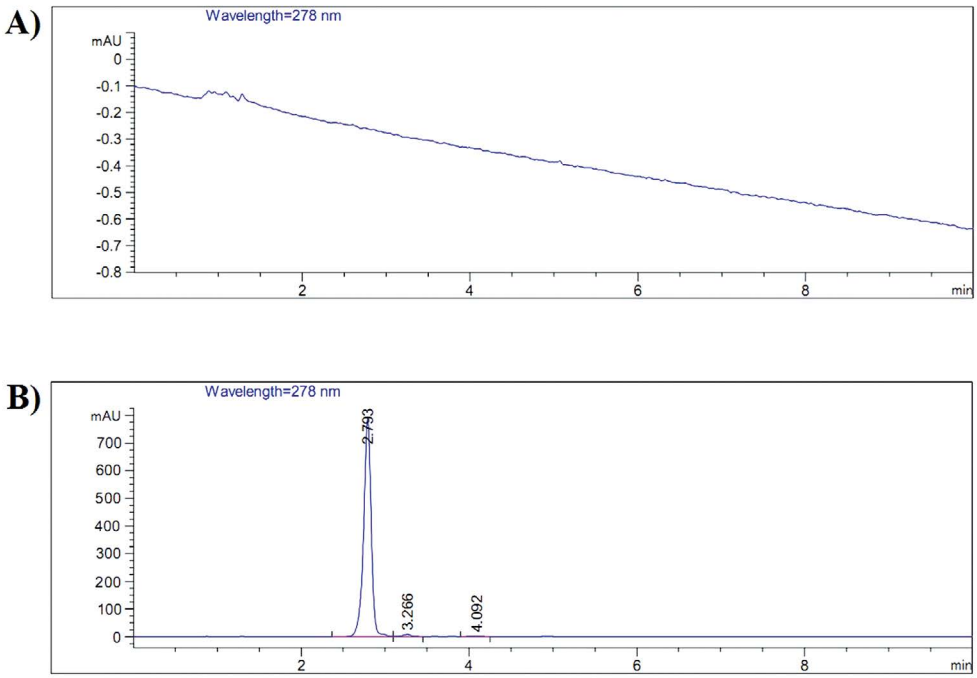

Figure 3. **Chromatograms of a solvent (A) and test solution (B) of T2**

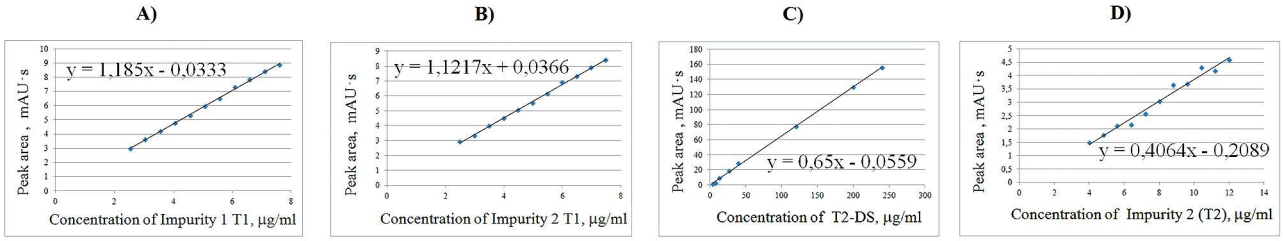

Figure 4. **Linearity between peak area and concentration of:**
A – Impurity 1 T1; B – Impurity 2 T1; C – T2-DS; D – Impurity 2 T2

"Precision" of the methods was estimated by parameters "repeatability" and "intermediate precision". "Repeatability" was confirmed by calculation of relative standard deviations (RSD ≤ 5 %) for the impurities determination results on the chromatograms of 6 different sample solutions of T1 and T2. "Intermediate precision" was confirmed by Fisher's and Student's tests, calculated from the peak areas on chromatograms of sample solutions of T1 and T2, obtained in different days on the same equipment and the same series of T1 and T2 samples. The validation results (Table 2) meet the acceptance criteria.

According to the range of experimental data, confirming the linear model, "Range" of the developed methods is 50–120 % of specification levels for Impurities 1 and 2 in T1 and for T2-DS and Impurity 2 in T2.

CONCLUSION

Methods for the determination of impurities in new promising antioxidants dodecyl(3,5-dimethyl-4-hydroxybenzyl)sulfide and bis-[3-(3,5-di-tert-butyl-4-hydroxyphenyl)propyl]sulfide were developed and validated during the research work. These methods may be included in normative documentation projects for the objects of study. It will promote future researches and registration of dodecyl(3,5-dimethyl-4-hydroxybenzyl) sulfide and bis-[3-(3,5-di-tert-butyl-4-hydroxyphenyl) propyl]sulfide as medicines.

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Table 2. Results of methods validation for the HPLC determination of relative substances in T1 and T2

Validation parameters	Determination of relative substances in T1		Determination of relative substances in T2	
Specificity	Chromatogram of solvent has no peaks at retention time of T1 and its impurities; R_{S1} = 6,35 и R_{S2} = 10,17		Chromatogram of solvent has no peaks at retention time of T2 and its impurities; R_{S1} = 2,74 и R_{S2} = 4,20	
System suitability	R_s = 24,25; N = 5306; A_s = 1,077; RSD% = 0,84 %		R_{s1} = 2,69; R_{s2} = 4,17; N_{T2} = 5465; $N_{T2\text{-}DS}$ = 3854; $N_{Imp.\ 2}$ = 1894; As_{T2} = 1,27; $As_{T2\text{-}DS}$ = 1,12; $As_{Imp.\ 2}$ = 0,89; $RSD\%_{T2}$ = 0,98 %; $RSD\%_{T2\text{-}DS}$ = 1,06%; $RSD\%_{Imp.\ 2}$ = 1,62 %	
Linearity	*r* = 0.9993 (Impurity 1)		*r* = 0,9997 (T2-DS)	
	r = 0.9992 (Impurity 2)		*r* = 0.9885 (Impurity 2)	
Precision (repeatability/ intermediate precision)	RSD = 0.98 % (Impurity 1)	F = 1,74 F (95 %, 5,5) = 5,05 t_{St} = 2,16 t_{St} (95 %, 10) = 2,23	RSD = 0.53 % (T2-DS)	F = 2,13 F (95 %, 5,5) = 5,05 t_{St} = 0,51 t_{St} (95 %, 10) = 2,23
	RSD = 1.22 % (Impurity 2)	F = 3,87 F (95 %, 5,5) = 5,05 t_{St} = 0,49 t_{St} (95 %, 10)= 2,23	RSD = 1.29 % (Impurity 2)	F = 1,24 F (95 %, 5,5) = 5,05 t_{St} = 2,04 t_{St} (95 %, 10) = 2,23
Accurace	t_a = 0.4396 (Impurity 1) t_{St} (95 %; 9) = 2.26		t_a = 0.18 (T2-DS) t_{St} (95 %; 6) = 2.45	
	t_a = 0.4382 (Impurity 2) t_{St} (95 %; 9) = 2.26		t_a = 1.21 (Impurity 2) t_{St} (95 %; 9) = 2.26	
Limit of quantification	0.65 mg/ml (Impurity 1)		12.89 mg/ml (T2-DS)	
	0.68 mg/ml (Impurity 2)		3.89 mg/ml (Impurity 2)	

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