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Rapid and sensitive LC separation of new impurities in trimethoprim

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Abstract

Trimethoprim is a chemotherapeutic often used in combination with sulfonamides. Herein, we report on the development and validation of a new HPLC assay of trimethoprim. The test allows the identification of new impurities that have not been detectable with any other known method including European Pharmacopoeia and USP. Trimethoprim and its impurities were eluted on a C18 column with a mobile phase consisting of methanol and a solution of sodium perchlorate at a flow rate of 1.3 ml/min and was quantified by UV detection at 280 nm. Overall, the method is simple, rapid and reliable for the detection of six impurities in trimethoprim batches.

Keywords: Reversed-phase chromatography; Trimethoprim; Impurities; Validation

1. Introduction

Herein, a newly developed LC method in order to determine impurities appeared in industrial batches of the chemotherapeutic trimethoprim (**1**) is described. Trimethoprim (**1**) is usually co-administrated with sulfonamides. Due to their synergistic effects on folic acid synthesis of bacteria, co-administration leads to a bactericidal effect. Therefore, resistance is seldom observed [1].

Combinations of trimethoprim (**1**) and sulfonamides are widely used for the treatment of cystitis, bronchitis, meningitis, and infections with pneumocystis carinii and therefore also in patients suffering from AIDS to suppress opportunistic infections [2].

Modifications in the manufacturing process have often come up after the loss of protection by patents. Manufacturers all over the world are interested in the synthesis of drugs out off patent protection and introduce manufacturing procedures more or less different from the original one. This was also the case for trimethoprim (**1**).

Because of these changes in the manufacturing procedures, new and unknown impurities can be

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observed in drugs. One of the most well-known drug catastrophes was based on undetected impurities: L-Tryptophan and one impurity. This contaminant, for example, has been reported to cause the severe disease eosinophilia-myalgia syndrome (EMS) [3]. Therefore, it is important to update the analytical methods of especially widely used drugs such as trimethoprim.

The observation of unknown impurities in trimethoprim discovered by The British Veterinary Medicines Directorate (VMD) put new scientific interest on trimethoprim. It was the aim of this study to investigate trimethoprim batches on impurities, pariticularly on related compounds. It was our goal to develop an isocratic, qualitative LC–UV method that is suitable for the detection of related substances in trimethoprim batches.

With the LC–UV method described herein, the separation of the possible process impurities **2**–**7** from trimethoprim (**1**) was achieved (see Fig. 1). The development of this method was important, because the impurities could not be detected with compendial methods, which have mainly focused on the identification of process intermediates such as 3-amino-(3,4,5-trimethoxybenzyl)acrylonitrile using thin-layer chromatography [4–6]. Our investigations led to a revision of the trimethoprim monograph in the European Pharmacopoeia, which has recently been published. [7] An Atmospheric Pressure Chemical Ionization (APCI) LC-MS method for the isolation and identification of the ethoxy derivative **3** and the bromo derivative **4** has also recently been described [8].

The LC–UV method described herein is validated with the following parameters: precision, accuracy, recovery, linearity and range, limit of detection, limit of quantification, and ruggedness of the method.

2. Experimental

2.1. Liquid chromatography system

The LC system includes a Bischoff HPLC pump, a 20 μl injection loop, a Bischoff UV detector LAMBDA 1000, a Shimadzu registrator C-R6A.

Chromatographic separation was achieved isocratically at ambient temperature in polymer covered reversed phase columns of the following type: Nucleosil 120-5, C18 AB, 250 mm × 4.6 mm I.D. with a pore diameter of 10 nm (Macherey-Nagel, Düren, Germany) was generally used.

The mobile phase consisted of 3 volumes of methanol and 7 volumes of a 1.4 g/l aqueous solution of sodium perchlorate monohydrate. The mobile phase was adjusted to pH* 3.1 with concentrated phosphoric acid and was degassed with ultrasound (15 min) before use. Flow rate was 1.3 ml/min.

The system was equilibrated for approximately 30 min. Trimethoprim (**1**) and its by-products were monitored at 280 nm, and 254 nm, respectively. Injection volume was 20 μl. The attenuation of the registrator was chosen between 6.0 (sensitive) and 8.0 (less sensitive).

2.2. Chemicals and reagents

All chemicals and reagents were of the highest possible purity. As standard trimethoprim CRS was used.

Diaveridine (**2**) was purchased by ICN (Eschwege, Germany) and was of 99.9% purity. Impurities 2,4-diamino-5-(4-ethoxy-3,5-dimethoxybenzyl)pyrimidine (**3**), 2,4-diamino-5-(3,4,5-trimethoxybenzoyl)pyrimidine (**5**), 4-amino-2-methylamino-5-(3,4,5-trimethoxybenzyl)pyrimidine (**6**), and 2-amino-4-methylamino-5-(3,4,5-trimethoxybenzyl)pyrimidine (**7**) were synthesized according to described methods [9–12]. The purity was determined using HPLC and was higher than 99.8%.

Impurity 2,4-diamino-5-(3-bromo-4,5-dimethoxybenzyl)pyrimidine (**4**) was obtained from the Bundesinstitut für Arzneimittel und Medizinprodukte (Federal Institute for Drugs and Medical Devices), Berlin, Germany and was of 97.5% purity.

Methanol was HPLC gradient grade quality and supplied by Merck (E. Merck, Darmstadt, Germany); Sodium perchlorate monohydrate was ASC standard quality and supplied by Aldrich (Steinheim, Germany). Ion exchanged water (UO

100, Letzner GmbH, Hückeswagen, Germany) was further purified by bidistillation. The aqueous solution of sodium perchlorate monohydrate was filtered off through a cellulose acetate filter (0.45 μm) before use.

2.3. Preparation of the standard solutions

Standard stock solutions were prepared separately for each batch (1 mg/ml) in the mobile phase. In some cases ultrasound was necessary

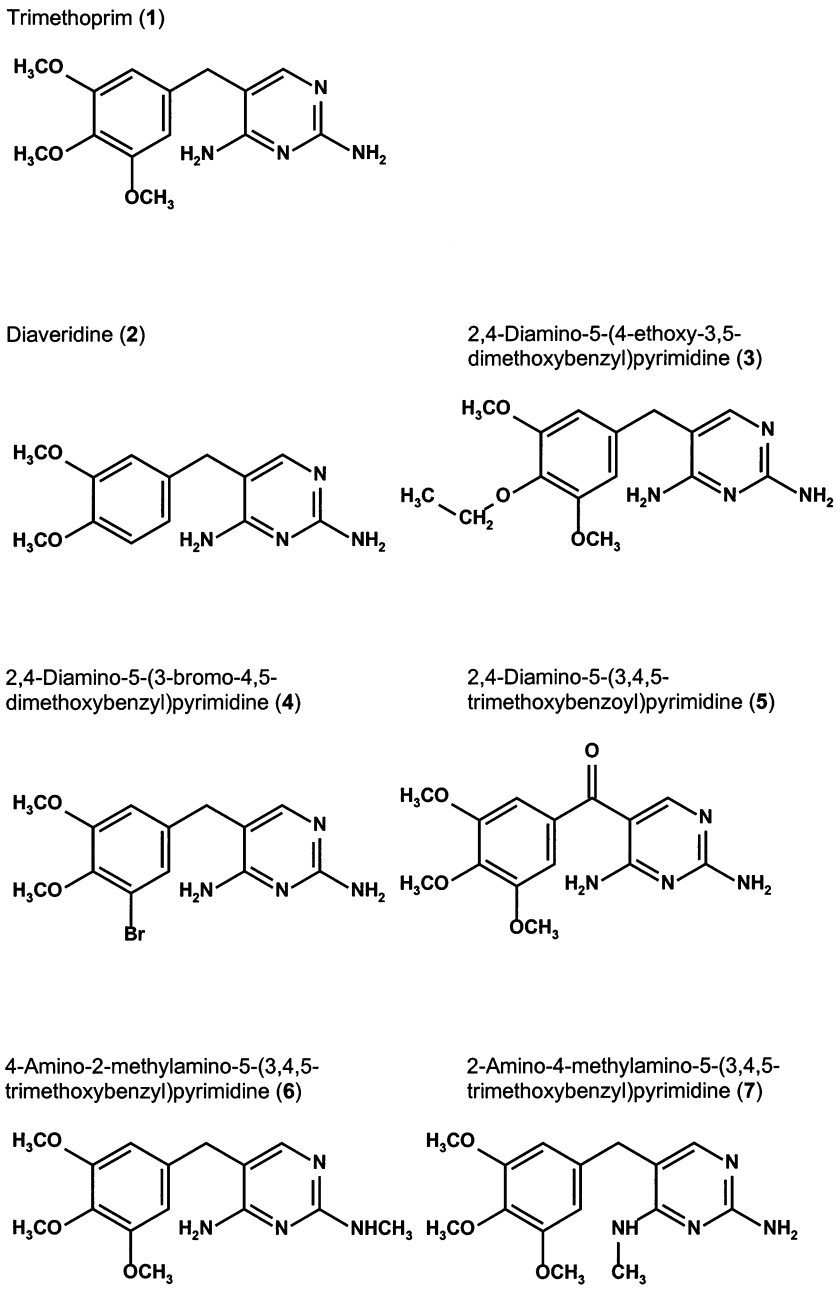

Fig. 1. Investigated compounds.

due to the observation of polymorph forms of trimethoprim (**1**) with different solution behavior. The solutions of the impurities were prepared analogously.

Calibration reference solutions were prepared by spiking the Chemical Reference Standard (CRS) solution with each standard stock solution of the investigated impurities in concentrations from 0.01 up to 1.5% of the nominal concentration of trimethoprim (**1**). Quantification was calculated from calibration curves.

The system suitability test was carried out before and after each sequence and consisted of 0.004 mg/ml trimethoprim CRS and 0.004 mg/ml 2,4-diamino-5-(4-ethoxy-3,5-dimethoxybenzyl)pyrimidine (**3**). The resolution of the two principal peaks had to be at least 5, and was determined according to the method of the European Pharmacopoeia with the following equation [7]:

$$Rs = 1.18 \times (t_{rb} - t_{ra})/b_{0.5a} + b_{0.5b}.$$

3. Results and discussion

3.1. Chromatography

In the described LC system an analytical method was developed to separate the following impurities from trimethoprim (**1**) (see Fig. 1):

- Diaveridine (**2**),
- 2,4-Diamino-5-(4-ethoxy-3,5-dimethoxybenzyl) pyrimidine (**3**),
- 2,4-Diamino-5-(3-bromo-4,5-dimethoxybenzyl) pyrimidine (**4**),
- 2,4-Diamino-5-(3,4,5-trimethoxybenzoyl) pyrimidine (**5**),
- 4-Amino-2-methylamino-5-(3,4,5-trimethoxybenzyl)pyrimidine (**6**), and
- 2-Amino-4-methylamino-5-(3,4,5-trimethoxybenzyl)pyrimidine (**7**).

The retention times are given in Table 1. Samples spiked with investigated impurities in concentrations of 0.1 and 0.3%, respectively, are shown in Fig. 2 and Fig. 3.

Diaveridine (**2**) was the only compound eluting before trimethoprim. It had a retention time of approximately 2.8 min. The remaining impurities were eluted after the trimethoprim peak. Some manufacturers were represented to illustrate the new impurity 2,4-Diamino-5-(4-ethoxy-3,5-dimethoxybenzyl)pyrimidine (**3**). Its retention time was about 5.6 min. The analytical data of the purified and isolated **3** were compared to that synthesized and have been proven to be identical.

Next to **3**, other impurities were investigated. 2-Amino-4-methylamino-5-(3,4,5-trimethoxyben-

Table 1
Retention times of the investigated compounds[a]

Compound	Retention times (min)	Relative retention times
Trimethoprim (**1**)	2.9	1.00
Diaveridine (**2**)	2.8	0.96
3	5.6	1.93
4	10.7	3.69
5	5.8	2.00
6	3.6	1.24
7	5.2	1.79

[a] The retention times of trimethoprim **1** and impurities **2**–**7** range from 2.8 to 10.7 min.

Table 2
Investigation of several industrial batches on impurities

Batch No.	HPLC-Results (Area under the curve in%, identified impurity[a])
1	2.9 min: Trimethoprim
2	2.9 min: Trimethoprim
3	2.9 min: Trimethoprim 5.6 min (0.90%, impurity **3**)
4	2.9 min: Trimethoprim 5.6 min (0.92%, impurity **3**)
5	2.9 min: Trimethoprim 5.6 min (1.2%, impurity **3**)
6	2.9 min: Trimethoprim 6.0 min: (0.95%, impurity **5**)
7	2.9 min: Trimethoprim 10.5 min (<0.1%)
8	2.9 min: Trimethoprim 5.8 min: (0.17%, impurity **3**) 10.9 min (<0.1%)
9	2.9 min: Trimethoprim 2.8 min (<0.1%) 11.2 min (<0.1%)

[a] Impurities below 0.1% remain unidentified.

zyl)pyrimidine (**7**) eluted at 5.2, the isomer 4-amino-2-methylamino-5-(3,4,5-trimethoxybenzyl) pyrimidine (**6**) at 3.6 min.

We have chosen the easy available 2,4-Diamino-5-(3,4,5-trimethoxybenzoyl)pyrimidine (**5**) as standard for the system test. Its retention time

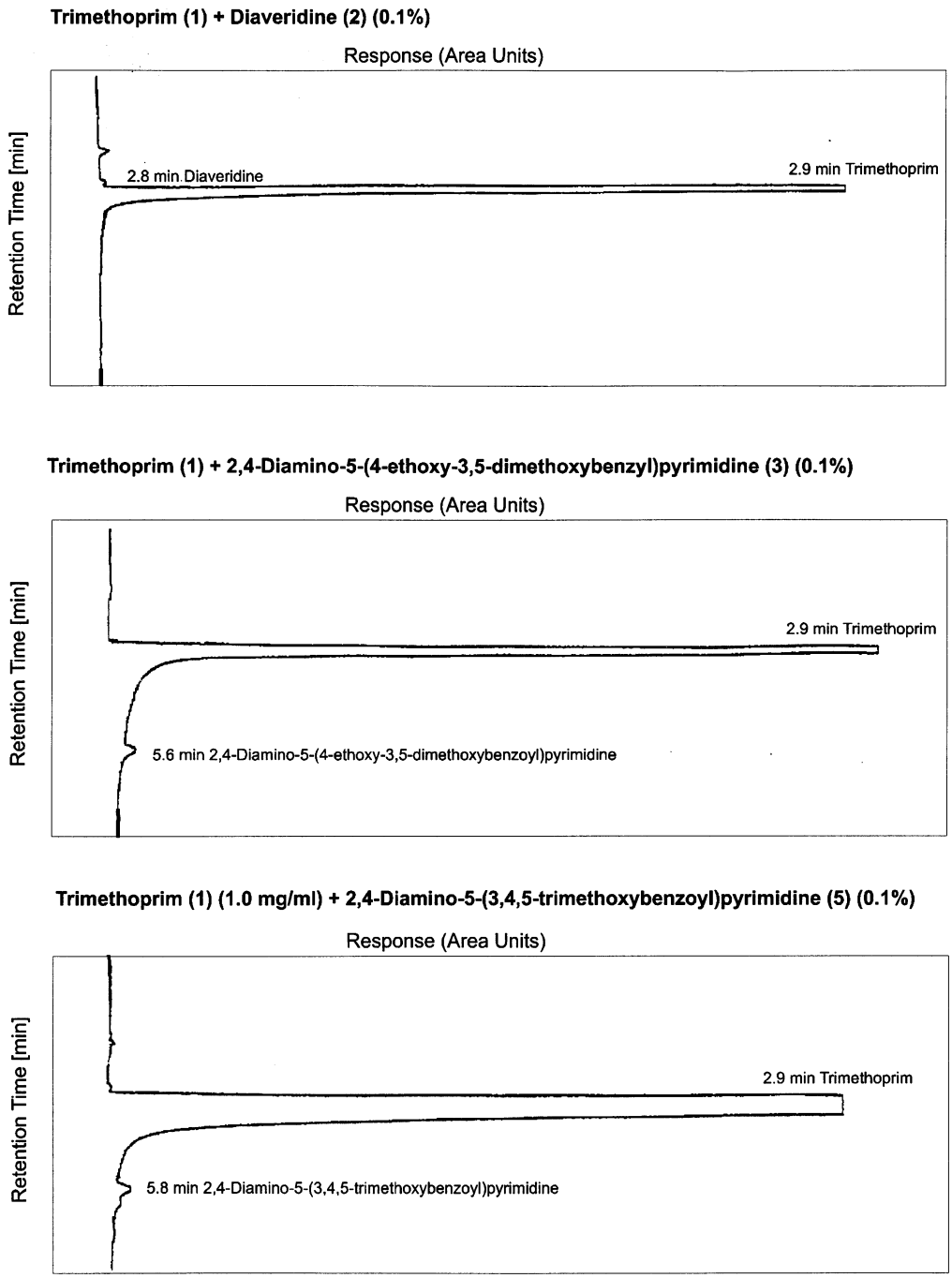

Fig. 2. Trimethoprim CRS, spiked with investigated impurities (0.1%).

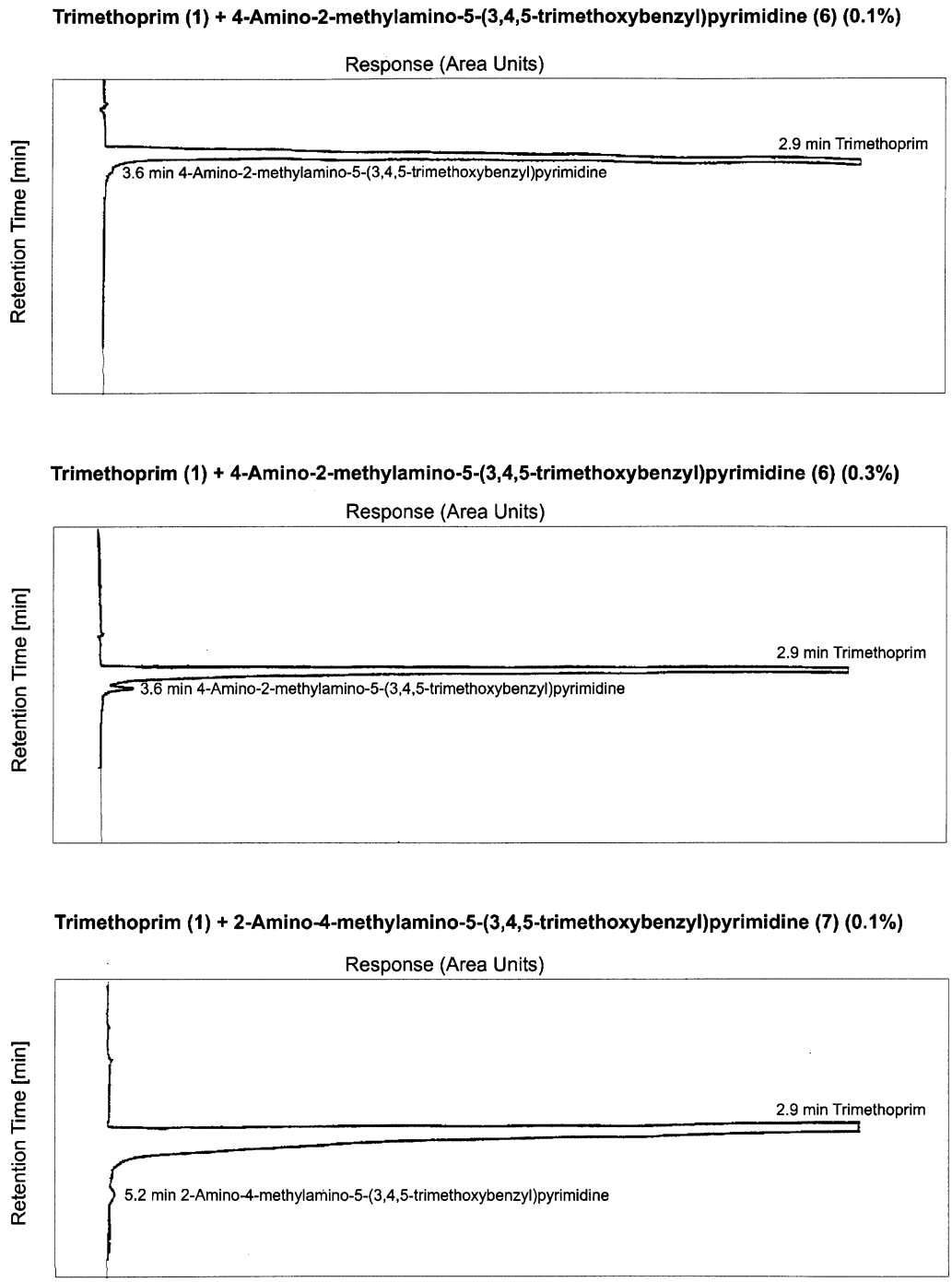

Fig. 3. Trimethoprim CRS, spiked with investigated impurities (0.1 and 0.3%).

was about 5.8 min and therefore closest to the so far unknown **3**. The bromo derivative **4** was also investigated, its retention time was 10.7 min. In contrary to the American market, this impurity was not found in batches available on the European market [8]. Table 2 shows representative batches, containing no impurity (batches 1 and 2), impurity **3** in variable amounts (batches 3–5, 8), impurity **5** (batch 6) and unidentified impurities smaller than 0.1% (batches 7–9).

3.2. Linearity

Quantification of trimethoprim and its impurities was based on the calibration of trimethoprim CRS and the impurities. After having checked the linearity of trimethoprim and its impurities, we have additionally checked the linearity of the by-products systematically added to a standard solution of 1.0 mg/ml trimethoprim.

Therefore, linearity of trimethoprim was checked ranging from 0.005 to 1.00 mg/ml. Spiked impurities were checked ranging from 0.1 to 2.1 μg/ml (corresponding to 0.01–0.2% referred to the nominal concentration of 1.0 mg/ml trimethoprim). It was statistically tested (significance level $\alpha = 0.05$) that the confidence intervals of all calibration curves enclose the origin.

3.3. Precision

The precision of the method was evaluated by injecting a trimethoprim solution ten times. The relative standard deviation (RSD) was 1.19%.

3.4. Accuracy

To determine the accuracy, 2,4-diamino-5-(3,4,5-trimethoxybenzoyl)pyrimidine (**5**) was added to a trimethoprim standard solution at 0.18% to 1.5% of the nominal concentration of trimethoprim. An average recovery of 96.1% was obtained for that range.

3.5. Limit of detection and Limit of quantification (LOD and LOQ)

The limit of detection and limit of quantitation of the impurities **3**, **5**, **6**, and **7** was calculated according to [13] from different concentrations of the by-products and the corresponding area counts. A summary of all limits of detection and quantification determined is given in Table 2. Despite of compound **6** which has a very weak UV absorption at 280 nm, the limit of detection could be determined in the range of 0.19–0.69 μg/ml and limit of quantification in the range of 0.29–1.02 μg/ml. The solution of impurity **6** showed a UV maximum at 254 nm. The LOD and LOQ was therefore also determined at 254 nm. The limit of detection at 254 nm was 0.25 μg/ml, and the limit of quantification was 0.37 μg/ml. Signal-to-Noise-Ratios of greater than 10 were observed for all described impurities at concentrations lower than 0.1 ug/ml. The method is therefore selective and sensitive to detect all described impurities (Table 3).

3.6. Ruggedness

In order to determine the ruggedness of the method, the stability of the trimethoprim solution was tested. Therefore, test solutions of six batches of trimethoprim were prepared according to the control test and stored. They were chromatographed at the beginning, after 24 h and 48 h. The solutions were stable during the investigated 48 h and the relative standard deviation was determined with 1.60–3.59%.

4. Conclusions

The present method was developed in order to identify new impurities in trimethoprim batches.

Table 3
Limit of detection and limit of quantification[a]

Compound Compound	2.60 (0.25 at 254 nm) Limit of detection (μg/ml)	3.78 (0.37 at 254 nm) Limit of quantification (μg/ml)
3	0.31	0.45
5	0.19	0.29
6	2.60 (0.25 at 254 nm)	3.78 (0.37 at 254 nm)
7	0.69	1.02

[a] The LOD and LOQ of **3**, **5**–**7** were determined at 280 and 254 nm, respectively.

Several statements can be made about the investigations of trimethoprim. First, the suitability of an isocratic method with UV detection for the determination of impurities in trimethoprim batches has been described. Secondly, the detection and quantification limits are indicating a reliable and very sensitive method. To enhance the sensitivity of compound **6**, it is recommended to determine impurities at 280 as well as 254 nm. We have identified batches with unacceptable high impurities around 1.0% of **3** which could not be found with common, compendial methods. The described method can be easily carried out. An expensive equipment for the determination of the impurities described herein is not necessary. Thus, guaranteeing highly purified samples in future times.

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