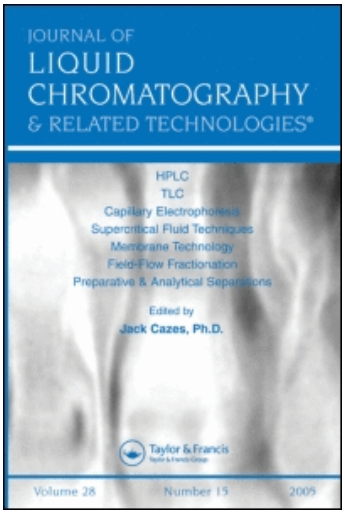

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DIRECT HPLC ANALYSIS OF TRIMETHOPRIM IN MILK

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DIRECT HPLC ANALYSIS OF TRIMETHOPRIM IN MILK

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ABSTRACT

A rapid assay has been developed for on-line SPE-HPLC determination of trimethoprim in milk. SPE precolumns packed with reversed-phase sorbent (RPS C-18) and with restricted access material (ADS C-18) have been tested in the column switching system. Only a simple preparation procedure for the milk sample is required before the direct injection. A Symmetry C-18 analytical column and mobile phase consisting of 100 mM phosphate buffer (pH3), with the addition of an ion-pair agent (PSA) and acetonitrile, have been used for HPLC analysis. UV detection was performed at 229 nm. The detection limit for trimethoprim in milk was 5 ng/mL.

INTRODUCTION

Trimethoprim (TMP, Fig. 1) is a synthetic antibacterial agent commonly used in connection with many animal species.(1). The residue of TMP in animal

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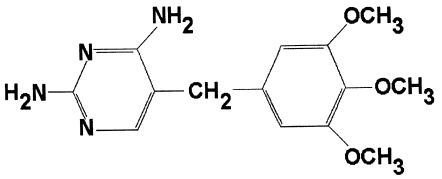

Figure 1. Chemical structure of trimethoprim.

samples could have an unpleasant effect on humans, especially those who are chronically ill, pregnant, or elderly. Maximal residual limit (MRL) for TMP in milk determined by the European Community is 50 ng/mL.(2)

Some authors published HPLC analysis of TMP, but only in clinical samples, such as plasma, serum, or urine.(3,4) HPLC-MS has also been applied for TMP analysis in bovine serum.(5) We have recently published the first paper dealing with HPLC analysis of TMP in milk and meat.(6) Milk samples were, after homogenization, mixed with McIlvaine buffer and the mixture was shaken and centrifuged. SPE C-18 cartridges, Sep-Pak C-18, were used for cleaning the extracts and TMP was eluted by the mixture of methanol and phosphate buffer with addition of an ion-pair agent. Extraction recovery was ca 73% for TMP in milk at the concentration level 50 ng/mL. TMP was analyzed on a Nova-Pak C-18 column with the mobile phase consisting of 12.5% acetonitrile and 87.5% phosphate buffer with the addition of PSA. TMP was detected by a DAD detector with dual detection (229 and 280 nm). HPLC analysis was realized in ca 15 minutes. The limit of determination for TMP 15 ng/mL was lower than MRL of the EU.

Only a few papers have recommended on-line SPE as the preparation of milk before HPLC analysis. The HPLC analysis of organochlorine pesticides in milk, using direct injection by the column-switching mode with ISRP (Internal Surface Reversed Phase) human serum albumin (HSA) precolumn, which was connected to the ODS Hypersil analytical column(7) was published. The milk sample was injected after dilution with the mobile phase (water-acetonitrile, 7:3) to 50% or 25% (v/v) milk solution. Concentrations of 50 μg/μL of pesticides were analyzed with extraction recoveries ca 99.3%.

The same authors have also published on-line extraction and determination of carbofuran in milk.(8) The diluted milk samples (5, 10, 15%) were injected directly into the HSA C-8 analytical column. The mixture of phosphate buffer and acetonitrile was used as the mobile phase. Milk proteins were eluted about 2 minutes before the peak of carbofuran (retention time 5.9 minutes). Recoveries were 98.4-102.7% in the concentration range 0.062-1.00 μg/mL. Detection limit was 0.025 μg/mL.

HPLC-integrated SPE with the photochemical post-column derivatization has been developed for the determination of oxacillin, cloxacillin, and di-

cloxacillin in milk.(9) The authors used the centrifugation step after the addition of acetonitrile for the fat removing and the ultrafiltration for protein removing. The photochemical post-column derivatization has possibilities for the analysis of very low concentrations of MRL values (30 μg/kg). Off-line SPE for these compounds in milk could also been applied,(10) but on-line procedure is much more effective for routine monitoring.

The aim of this paper was to present the possibility for the direct HPLC analysis of TMP in milk samples without any complicated preparation steps. Both C-18 SPE and RAM precolumns have been tested in the column-switching method for TMP determination at a concentration level of MRL values.

EXPERIMENTAL

Chemical and Solutions

Standard TMP (Fig. 1) was obtained from Sigma (USA), pentanesulphonic acid (PSA) from Altech (USA), acetonitrile, methanol (HPLC grade), and phosphoric acid (p.a.) were supplied by Merck (Germany) and $NaH_2PO_4.2H_2O$ (p.a.) by Chemapol (CZ). Milk was from a grocery store in Bratislava (Slovakia).

Stock standard solutions of TMP (137 μg/mL) was prepared in methanol and stored in the refrigerator at 5°C. Working solutions were prepared by diluting stock solution with deionized water to prepare solutions containing 50-500 μg TMP/mL.

Equipment

The column-switching system consisted of an HP 1100 HPLC system (Hewlett Packard, USA), a pump 64 (Knauer, Germany), an injector U6K (Waters, USA), a switching valve P/N 60057 (Waters, USA). A laboratory micro-shaker ML1 (Poland), a centrifuge MPW -300 (Mechanika precyzyjna, Poland), and a sonicator Sonorex RK100H (Bandelin electronic, Germany) were used for the sample preparation. A Separon RPS C-18 cartridge (30×3.3mm; 5μm), Tessek (Czech Republic)) and LiChrospher RP-18 ADS (25×4mm; 25μm), Merck (Germany) were tested as SPE precolumns. A Symmetry C-18 column (150×3.9mm; 5μm), Waters (USA) was used as an analytical column.

Chromatographic Conditions

The column-switching system was arranged according to the scheme in Fig. 2. Deionized water was used as a washing solution for SPE precolumns –

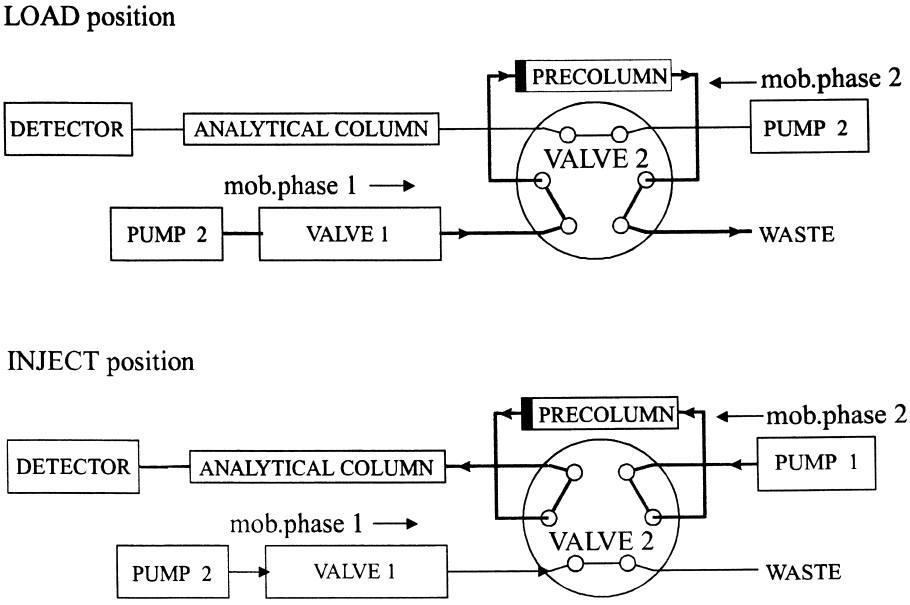

Figure 2. Column-switching scheme.

mobile phase 1, (F=1.0 mL/min) and mobile phase 2 consisted of 100 mM phosphate buffer, adjusted to pH 3, with 100 mM PSA (95:5) and acetonitrile (85:15 or 88:12) with flow rate 0.8 mL/min. Detection was realized at 229 nm.

Sample Preparation

Homogenized milk was centrifuged for 30 minutes at 3000 rpm and the middle layer was injected into the switching system (volume 50 μL). Some samples were diluted with water or the mobile phase to 50% milk solution.

RESULTS AND DISCUSSION

Trimethoprim is a basic compound showing relatively strong interactions of amino-groups with the residual silanols of the reversed-phase packing material, therefore, an addition of ion-pair agent (PSA) into the mobile phase was necessary for better symmetry of the peak. The assymetry factor for TMP standard

using the Symmetry C-18 column was less than 1.3. As was found in our previous work, the detection at 229 nm is convenient for TMP analysis. If effective sample preparation is applied, no interfering compounds are present in the chromatogram at this wavelength.

In this paper, the column-switching chromatographic system using ADS RP-18 and RPS C-18 precolumns has been applied for the direct analysis of TMP in milk samples. As has recently been published,(11) for the use of ADS precolumns, no preparation of milk sample is necessary before the injection. The milk sample was only centrifuged and, if necessary, diluted 1:1 with water or the mobile phase. This fact was also confirmed by the other authors.(12) They considered the centrifugation of milk sample as effective as, e.g., extraction of fat by n-hexane. Upon centrifugation of milk, three distinct layers were formed. The top layer was fat, and precipitated proteins formed the bottom layer. The middle layer was injected into the column-switching system and the analyte was backflushed from C-18 precolumn into the analytical column (Fig. 2).

The various washing eluents were tested for removing the rest of the interfering compounds from SPE precolumns; and pure water without any organic modifier seems to be the best solution. The addition of organic solvent into the washing solutions could cause the problem of back pressure of the system; the precipitation of the resting proteins from milk in the precolumn has also been observed. Moreover, most parts of proteins and lipids were removed by the centrifugation step.

The timetable of the complete TMP analysis by the column-switching system, which is illustrated in Table 1, was developed for RPS precolumn, and it has also been applied for RAM precolumn. It was found out, that 10 minutes time for a washing step was sufficient for removing weakly retained compounds from precolumns. After that, the valve was switched to the injection position to transfer the analyte from the precolumn to the analytical column (Symmetry Shield C-18). A time of 2 min was adequate for a complete transfer of TMP. Subsequently, the valve was switched back and the precolumns were washed to remove strongly retained compounds and prepare it for the next analysis. No memory efect was observed using the same precolumn several times. For RPS a minimum of 10

Table 1. Timetable of the Complete TMP Analysis by the Column-Switching System

Time	Autosampler	Valve	Action
0	inject	load	injection of the 1st sample, cutting of the impurities
10		inject	transfer of the analyte
12		load	recondition of the precolumn
20	inject	load	injection of the 2nd sample

times, for RAM minimum of 25 times. After this time, chromatographic characteristics were not changed (k, As). For RPS precolumn capacity factor, k of TMP in milk was 3.89 min (RSD=0.5%), coefficient of assymetry As was 1.03 (RSD=2.3%), RAM precolumn k = 2.71 min (RSD=0.7%), As=1.55 (RSD= 1.9%). Repeatibility was measured by a 2-fold injection of three parallel extractions of milk samples, and determined as relative standard deviations (RSD).

Detection limit for TMP in milk at signal/noice ratio 3 was about 5ng/mL with detection at 229 nm. Extraction recoveries were determined as follows: ADS precolumn: 85% for the concentration 68.5 ng/mL and 89.4% for 685 ng/mL, RPS precolumn: 95.6% for the concentration of 68.5 ng/mL.

HPLC chromatograms of the analysis of milk diluted 1:1 and milk spiked with TMP at the concentration of 250 ng/mL on the ADS precolumn, is shown in Fig. 3a, b. It is possible to see that a small peak with the same retention time is

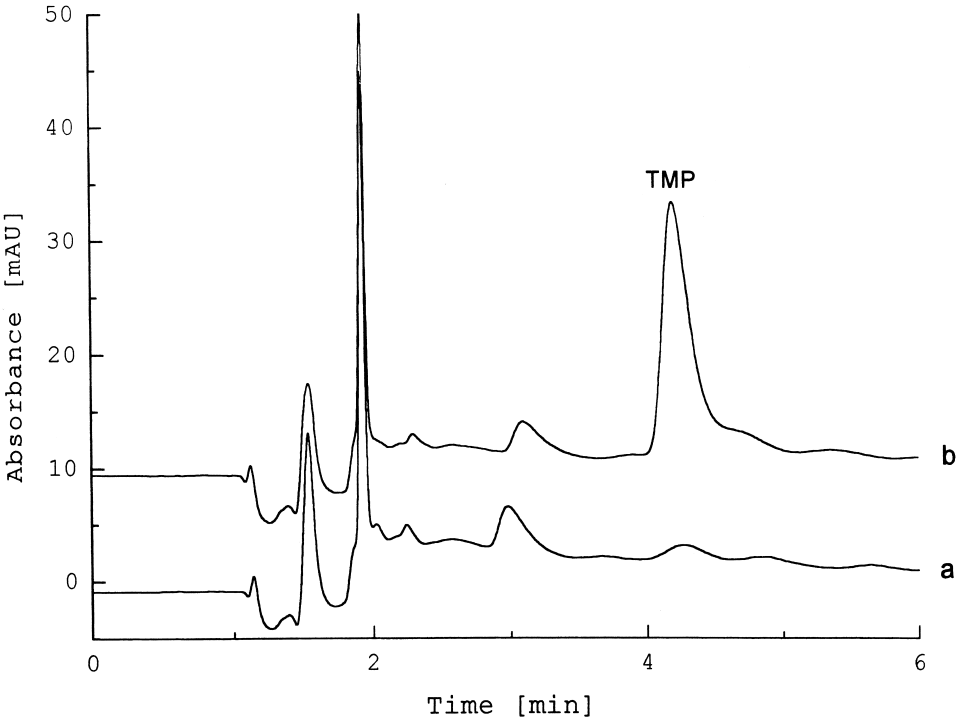

Figure 3. HPLC chromatogram of milk sample without and with TMP addition, ADS precolumn. Chromatographic conditions: Symmetry C-18 column (150 × 3.9 mm, 5µm), Precolumn: LiChrospher RP-18 ADS (25 × 4 mm, 25µm), washing eluent: water, mobile phase: 100 mM phosphate buffer (pH 3) with 100mM PSA (95:5), acetonitrile (88:12), flow-rate 0.8 mL/min. a) milk without TMP addition. b) milk with TMP addition (250 ng/mL), sample diluted 1:1.

present in the chromatogram. Its UV spectrum obtained with a DAD detector was the same as for TMP. The concentration of TMP in this peak was about 9.7 ng/mL. Only very small amounts of interfering compounds are present in the chromatogram, and the peak of TMP was evaluated without problems, for a low concentration level (9.7ng/mL).

The linear dependence of peak areas on TMP concentration ($y = ax + b$) was as follows : a=6.97, b=0.20, r=0.9995.

This small peak has also been seen in the HPLC chromatogram with RPS precolumn (Fig. 4a, b). It is obvious, that small amounts of TMP could be present in analyzed milk samples, but this concentration is lower significantly than the MRL value. In Figs. 4a, b, it is possible to see that much more interferences

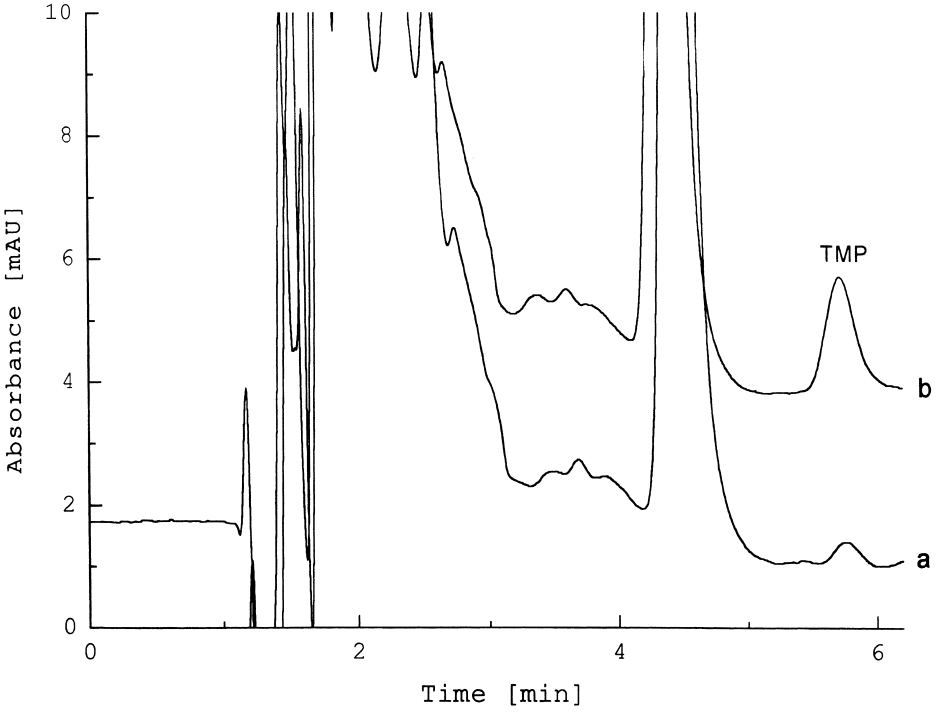

Figure 4. HPLC chromatogram of milk sample without and with TMP addition, RPS precolumn. Chromatographic conditions: Symmetry C-18 column (150 x 3.9 mm, 5µm). Precolumn: Separon RPS C-18 (30 x 3.3 mm, 5µm), washing eluent: water. Mobile phase: 100 mM phosphate buffer (pH 3) with 100mM PSA (95:5), acetonitrile (85:15), flow-rate 0.8 mL. a) milk without TMP addition. b) milk with TMP addition (68.5 ng/mL).

were present in the chromatogram, but the peak of TMP is separated without a problem of the rest of the compounds from milk matrix. The HPLC chromatograms with applications of both SPE precolumns could demonstrate, that the use of ADS precolumn provides a more "clean" chromatogram; this means that much more interfering components have been washed from the precolumn, especially large polar molecules eluting at lower retention times. This fact, could be explained by the character of the ADS precolumn. It is packed with the RAM sorbent, which restricts the access of large molecules, as proteins or lipids into a sorbent, and they are washed dominantly from the precolumn during the washing step.

The RPS precolumn is packed with the classical reversed-phase sorbent and more interfering compounds are retained, together with the analyte at the precolumn, and they have been not washed completely with the washing eluent. For these reasons, the RPS precolumn was washed after 10 injections of milk samples, RAM precolumn after 25 injections. But it was found, that k and As values did not change after more injections. When diluted milk samples (1:1) were injected, RPS precolumn was washed after 20-25 injections. Both RPS and RAM precolumns were applied without the method degradation. More than 70 injections for RPS and 50 injections for RAM precolumns were realized without the change of chromatographic parameters. As RAM precolumn has only been tested for the application in this assay, this precolumn will be used for the next analyses in the future.

A selective column-switching method for the determination of TMP was developed and sample clean-up procedures, using both RAM sorbent and the conventional RP precolumns, were compared. It was demonstrated, that the use of ADS sorbent provides more selective removing of interfering proteins and endogenous compounds from the milk sample. If diluted milk samples were injected, a two times higher number of direct injections was realized.

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REFERENCES

1. Brandšteterová, E.; Kubalec P.; Bovanová L. *HPLC Determination of Antimicrobial Residues in Edible Animal Products, in Food Analysis by HPLC*; Nollet, L.M.L., Ed.; Marcel Dekker, Inc.: New York, 2000; 621-693.

2. Heitzman R.J.; *Veterinary Drug Residues. Residues in Food Producing Animals and Their Products; Reference Materials and Methods*, 2nd Ed.; Blackwell Scientific: Oxford, 1994.
3. Nordholm L.; Dalgaard L. Determination of Trimethoprim Metabolites Including Conjugates in Urine Using HPLC with Combined UV and Electrochemical Detection. J. Chromatogr. **1984**, *305*, 391-399.
4. Erdmann G.R.; Canafax D.M.; Giebink G.S.; Hplc Analysis of Trimethoprim and Sulfamethoxazole in Microliter Volumes of Chinchilla Middle Ear Effusion and Serum. J. Chromatogr. **1988**, *433*, 187-195.
5. Nachilobe P.; Boisson J.O.; Cassidy R.M.; Fesser A..C. Determination of Trimethoprim in Bovine Serum by HPLC with Confirmation by Thermospray Liquid Chromatography Mass-Spectrometry. J. Chromatogr. **1993**, *616*, 243-252.
6. Brandsteterova E.; Kubalec P.; Machakova L. HPLC Determination of Trimethoprim in Meat and Milk with an SPE Preseparation Procedure. Z. Lebensm. Unters. Forsch. **1997**, *204*, 341-344.
7. Menezes M.L.; Felix G. Analysis of Organochlorine Pesticides in Plain Milk Using Direct Injection on ISRP Column, with Column Switching. J. Liq. Chromatogr. **1996**, *19* (19), 3221-3228.
8. Menezes, M.L.; Felix G.; Demarchi, A.C.C.O. On-Line Extraction of Carbofuran in Raw Milk by Direct HPLC Injection on an ISRP Column. Chromatographia **1998**, *47* (1/2), 81-83.
9. Ibach A.; Petz M. HPLC-Integrated Solid-Phase Extraction with Photochemical Post-Column Derivatization for the Determination of Oxacillin, Cloxacillin, and Dicloxacillin in Raw Milk. Z. Lebensm. Unters. Forsch. **1998**, *207*, 170-173.
10. Kubalec P.; Brandsteterova E.; Bednarikova, A. Determintaion of Oxacillin, Cloxacillin, and Dicloxacillin in Milk, Meat, and Cheese Samples Using HPLC and Precolumn Derivatization. Z. Lebensm. Unters. Forsch. **1997**, *205*, 85-88.
11. Boos K.S.; Rudolphi, A. The Use of Restrictected Access Media in HPLC, Classification and Review. LC-GC **1998**, *11* (2), 84-95.
12. Dadgar D.; Power A. Application of Column-Switching Techniques in Biopharmaceutical Analysis. HPLC Determination of Tripelennamine in Bovine Plasma and Milk. J. Chromatogr. **1987**, *421*, 216-222.

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