Acta Poloniae Pharmaceutica – Drug Research, Vol. 77 No. 5 pp. 707–716, 2020
DOI: 10.32383/appdr/127474
Received 24 June 2020, Received in revised form 25 August 2020, Accepted 11 September 2020.
ISSN 2353-5288
Polish Pharmaceutical Society

ANALYSIS

DETERMINATION OF TIOACETIC ACID IN MESO-2,3-DIMERCAPTOSUCCINIC ACID

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Abstract: A selective, sensitive isocratic RP-HPLC method determination of tioacetic acid in meso-2,3-dimercaptosuccinic acid (DMSA) based on ion-pair interaction was developed. Validation of the procedures: selectivity, the limit of detection (LOD), limit of quantitation (LOQ), linearity, precision, accuracy, robustness, and system suitability parameters are presented. The excellent linearity between peak area and TOA concentration in the range of 0.013-0.60% was obtained. The LOD values of 0.001% or 0.0005% with respect to DMSA concentraton, depending on the used HPLC system, were obtained in optimized condition.

Keywords: HPLC, validation, DMSA, ion-pair, tioacetic acid, TOA

Meso-2,3-dimercaptosuccinic acid (DMSA) is a basic chelator agent for the treatment of heavy metals (especially lead but also mercury and arsenic) poisoning (1, 2) and an important ingredient of radiopharmaceutical kit used in scintigraphy of kidney (3). The quality control of this radiopharmaceutical kit is described in European Pharmacopeia (4). The comparison of quality control methods DMSA complexes of ^{99m}Tc and ^{188}Re used in diagnostics and therapy have also been described (5). Determinations of DMSA in human urine (6) and its metabolism in the human body (7) was investigated many years ago. Although synthesis of DMSA in the reaction of tioacetic acid (TOA) with acetylenedicarboxylic acid (ACDCA) (8, 9) was reported more than 60 years ago, the literature that concerns its quality control is very limited. One of the last studies is concerned with LC-MS separation and identification of forced degradation products of DMSA (10). The determination of ACDCA in DMSA has been described recently (11) but it's difficult to find literature about the determination of TOA in DMSA. Furthermore, only a few examples of separation and determination of TOA in drug substances are reported in the literature. Separation of tioacetic acid and other intermediates from drug substance captopril by using HPLC was described in 1981 (12). However, the method was used only for the determination of assay of the drug substance. Determination of tioacetic acid as one of the possible impurities in a racecadotril drug substance is described in European Pharmacopeia (13). Tioacetic acid has a very unpleasant odor and compared with other potential DMSA impurities like mercaptosuccinic acid (MSA), oxalic acid (OxA), or acetic acid (there is no toxicity data for ACDCA) is quite toxic. Acute toxicity (LD 50 for an intraperitoneal route in mouse) of TOA, MSA, and OxA are 75 mg/kg, 200 mg/kg, and 270 mg/kg, respectively (14).

The aim of this work was to develop and validate a method for separation and determination of thioacetic acid (TOA) in a meso-2,3-dimercaptosuccinic acid (DMSA).

EXPERIMENTAL

Chemicals and reagents

Deionized water (resistance above 18 MΩ) was obtained from a double Millipore system. Acetonitrile (ACN) was HPLC grade. All other reagents are analytical reagents grade. ACN and octylamine (OA) were obtained from Sigma-Aldrich, ortho-Phosphoric acid (H_3PO_4; 85.1%) and Sodium hydroxide from Chempur (Poland),

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Tioacetic acid (98%) and Acetylenedicarboxylic acid from Acros Organics. Mercaptosuccinic acid and oxalic acid were provided by Aldrich. Meso-2,3-dimercaptosuccinic acid (DMSA) was obtained from Chemieliva (China) and was purified in POLATOM. Some experiments were made by using ortho-Phosphoric acid (85%) HPLC purity obtained from Merck. Nitric Acid (65%) Suprapur®, ICP multi-element standard solution IV, (23 elements in diluted nitric acid) 1000 mg/L: Ag, Al, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Sr, Tl, Zn Certipur® were obtained from Merck.

Instrument and chromatographic condition

The chromatographic systems consisted of the HPLC pumps, UV detectors, column heaters, and autosamplers. Two systems were used: Perkin Elmer Flexar with PDA detector controlled by Total Chrom V6.3.2 software (HPLC 1) and Waters with PDA 2998 detector controlled by Empower 3 data system (HPLC 2). The analysis was carried out on three RP C18 analytical columns – Kromasil C18 RP; 100 × 4.6 mm, 5 µm – each packed the same stationary phase – Kromasil 100-5-C18. Column 1 and column 2 were manufactured from the same batch of packings and column 3 from the other one. A 2 µm inlet filter was placed before the column inlet and the column was kept at a temperature of 30°C, during analysis.

The mobile phase consisted of water-acetonitrile-octylamine (the pH of OA in H_2O was adjusted with phosphoric acid to 7.8-8.0) with ratio: 90 : 10 : 0.05. The analysis was carried out in isocratic condition at flow rate 1 mL/min and the injection volume was 20 µL. The detection wavelength was set to 245 nm and autosampler temperature was set to 20°C.

ICP analysis of metal impurities

The ICP analysis was performed using Perkin Elmer ICP-OES spectrometers 7300DV and 3300XL, both in axial viewing mode controlled by WinLab32 (Perkin Elmer) software. RF generator power was set to 1300 W. Number of replicates was set to 3 and the sample flow rate was 1.5 mL/min. Between every calibration standard and every sample, the spectrometers were rinsed with purified water for at least 3 min with a flow rate of 4.0 mL/min. Wavelengths of the tested elements were chosen as follows: Al 396.153 nm, B 249.772 nm, Ba 230.425 nm, Ca 317.933 nm, Cd 214.440 nm, Cr 284.325 nm, Cu 327.393 nm, Fe 238.204 nm, Mg 279.077 nm, Mn 259.372 nm, Ni 231.604 nm, Pb 220.353 nm, Si 251.611 nm and Zn 213.857 nm. The calibration curve was performed using approximately 0.5 mol/L HNO_3 as a blank, and standard solutions of mentioned elements in the range of concentration between 0.1 µg/mL to 1.0 µg/mL, diluted with the same solution of approximately 0.5 mol/L HNO_3.

Preparation of solutions

Ortho-phosphoric acid 0.054 M

Weighted accurately 6.22 g of phosphoric acid was dissolved in 1 liter of water in a volumetric flask**.**

Buffer pH 11.0-11.2 for samples preparation

To a 1 liter volumetric flask 345 mL of 0.2 M sodium hydroxide, the appropriate volume of 0.054 M orthophosphoric acid solution to obtain pH about 11.0 and then 0.6 mL of octylamine was transferred and the mixture was diluted to volume with water.

Stock solution of thioacetic acid (TOA)

To a 10-mL volumetric flask, 38.3 mg of tioacetic acid was weighed, dissolved, and diluted to volume with buffer.

Other solutions of TOA

Tioacetic acid solution 50 µg/mL was prepared from a stock solution of TOA by appropriate dilution of stock solution. This solution was stable for 8 hours at a temperature of 22°C.

Tioacetic acid solutions for calibration graph were prepared in the range of 0.125-6.00 µg/mL by appropriate dilution of 50 µg/mL solution with buffer.

Stock solutions of ACDCA, MSA and OxA

These stock solutions were obtained by weighting to 10 mL volumetric flasks appropriate amount of each acid i.e. ACDCA (4.4 mg), MSA (6.6 mg) and OxA (7.9 mg). Each weighed amounts of these substances were dissolved and diluted to volume with buffer.

Solution of mixture TOA, ACDCA, MSA, OxA and DMSA.

A solution containing 5 µg/mL of TOA, 100 µg/mL of ACDCA, 100 µg/mL of MSA, and 100 µg/mL of OxA was obtained by appropriate dilution of stock solutions with buffer.

Then the appropriate weighed amount of DMSA was dissolved in this solution to obtain final concentrations of DMSA – 1 mg/mL, 1 µg/mL of TOA, 100 µg/mL of ACDCA, 100 µg/mL of MSA and 100 µg/mL of OxA

Unspiked samples of DMSA (1 mg/mL)

To a 10 mL vial, about 5 mg of a meso-2,3-dimercaptosuccinic acid (DMSA) was accurately weighed. Then the appropriate volume of buffer was added by using a precise micropipette to obtain the concentration of DMSA 1 mg/mL and vial content was shaken. Usually, samples were quite good dissolved in buffer, if not the ultrasonic bath was used.

Samples of DMSA (1 mg/mL) spiked with TOA

To 10 mL vials was weighted about 5 mg of DMSA samples and then the appropriate volume of TOA standard solutions was added to obtain a final concentration of DMSA 1 mg/mL and appropriate concentration of TOA.

ICP-OES analysis of metal impurities

Analysis of stationary phase

Column conditioning

Kromasil C18 column used for the analysis of TOA in DMSA (after about 450 injections of samples) were conditioned by mobile phase (0.05% OA/H_3PO_4, pH = 8, 10% ACN) overnight at flow rate 0.25 mL/min. About 450 mL of the mobile phase has been pumped through the column in this way.

Preparation of samples for ICP-OES analysis

Two samples (26.4 mg and 20.8 mg) of stationary phase from this column were weighted to 10 mL vials and then an appropriate volume of 0.5 M nitric acid (i.e. 1.320 and 1.040 mL, respectively) to obtain a final concentration of samples about 20 mg/mL was added by precise micropipette. Then both samples were left for the next day and sonicated in an ultrasonic bath for a few minutes and finally filtered through 0.45 µm PTFE syringe filters (Whatman). Before ICP analysis both samples were diluted 20 times by 0.5 M nitric acid. Samples of the mobile phase before and after column conditioning, before ICP analysis, were diluted 20 times by 0.5 M nitric acid. Samples of concentrated ortho-phosphoric acid (85%) and octylamine used for the preparation of the mobile phase were diluted 50 times by 0.5 M nitric acid before ICP analysis.

RESULTS AND DISCUSSION

Preliminary experiments and method optimization

Tioacetic acid, DMSA and other impurities potentially present in DMSA are small, polar compounds that are difficult to separate on the ODS column using simple RP-HPLC mobile phases. It was therefore decided to use the ion-pair mechanism of separation. Octylamine (OA) has been chosen as a cheap and effective ion pair reagent. The starting composition of the mobile phase contains 0.1% of OA in 10% of ACN and the pH of the eluent was established by diluted ortho-phosphoric acid. Columns of different lengths (50, 100, 150, and 250 mm – all 4.6 ID and 5 µm particle size, contains the same stationary phase) were tested and finally, the 100 mm was chosen as the optimal. On the shortest column separation of TOA signal was not satisfactory, on the longest column the time of analysis would be very long- due to the large retention time

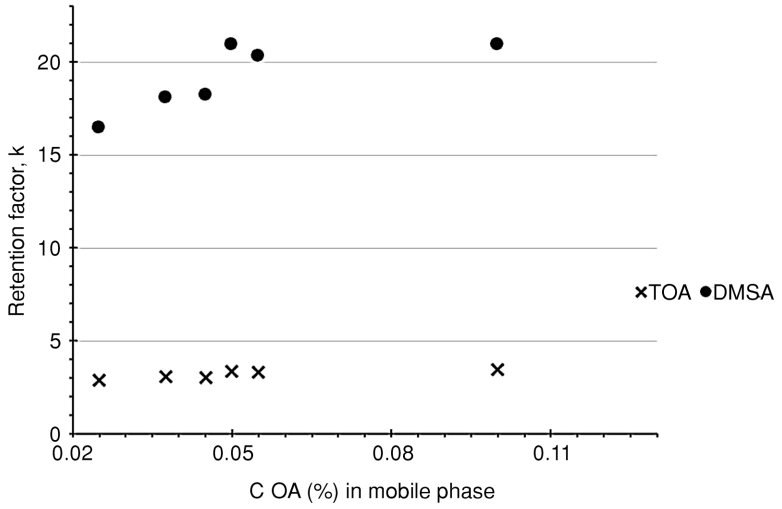

Figure 1. Effect of octylamine concentration in the mobile phase on the retention of TOA and DMSA.

of DMSA ion pair signal. Only columns of 100 and 150 mm allow for a good separation of TOA and elute of DMSA ion pair signals in time below 30 minutes. The pH of the mobile phase in the range of 7.2-8.3 does not impact the separation and retention time of the TOA. The next important factor which affects the separation and stability of the TOA signal is the concentration of OA in the mobile phase. The concentration of OA between 0.025-0.10% in the mobile phase has been tested and as is shown in Figure 1 the retention factors of both, TOA and DMSA are increased when OA concentration increase up to 0.05% and then there are stable.

When concentrations of OA in the mobile phase decrease to 0.038% and 0.025%, the TOA signal in spiked DMSA samples slowly decreases – 5.6% and 9.7%, respectively after about two and half hours after preparation. The important factors which determine the stability of the TOA signal in spiked DMSA samples are the pH of the sample and the presence of octylamine. When the DMSA sample spiked with TOA is dissolved directly in the mobile phase, the pH of the sample is too low and the TOA signal decreases in time. The optimum pH range of the analyzed sample is between 7.7 and 9.0. When the pH of the sample is above 9.0, the TOA signal is stable, but the baseline getting worse and integration of its signal is sometimes problematic. To obtain appropriate final pH, the sample (1 mg/mL of DMSA) should be dissolved in a mixture of 0.069 M NaOH and about 0.027 M H_3PO_4 with 0.06% of octylamine. The pH of this solution is usually between 11.0-11.2. The addition of octylamine to the NaOH/H_3PO_4 buffer caused the faster formation of the stable TOA signal. However, on the chromatograms of the sample dissolved in a mixture of 0.10% OA with NaOH/H_3PO_4 (pH 11.1) the peak before TOA is very unsymmetrical, which can cause a problem with the integration of the analyte signal. In optimized conditions, samples are injected 30 minutes after preparation on the column. Based on the UV spectrum obtained from the PDA detector it was found that TOA has an absorption maximum at 245 nm and this wavelength was chosen for its detection.

Method validation

Validation has been carried out according to ICH recommendation (15).

Specificity

Specificity was demonstrated by registering of chromatograms of diluent, DMSA, TOA, ACDCA, MSA, and OxA individually, and finally, DMSA sample spiked with TOA, ACDCA, MSA, and OxA. The obtained chromatograms are presented in Figure 2.

As is clearly evident the resolution between signals of TOA (as TOA ion pair) and neighbors signals are very good. Also, the separation between ACDCA and DMSA is good enough to distinguish between signals of the ACDCA –OA ion pair and the DMSA-OA ion pair.

System suitability test

System suitability parameters (SST) were measured to verify the systems, method and columns performance. The results of SST tests car-

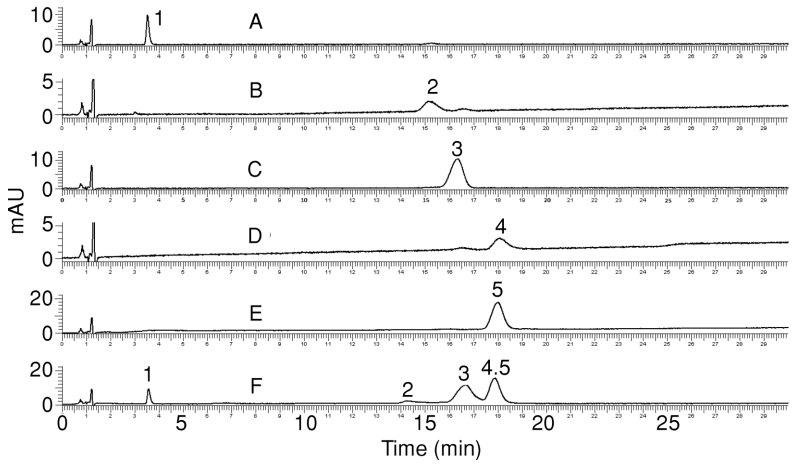

Figure 2. The example chromatograms of: TOA (1 µg/mL)- A, OxA (100 µg/mL) – B, ACDCA (100 µg/mL) – C, MSA (100 µg/mL) – D, DMSA (1 mg/mL) -E and mixture of all acids – F, registered at 245 nm. Signals: 1 – TOA, 2 – OxA, 3 – ACDCA, 4 – MSA and 5-DMSA.

ried out on two columns and two HPLC systems are presented in Table 1.

Better results are obtained on the HPLC 2 and column 3. However, all tested parameters for both systems fulfill the recommendations of European Pharmacopoeia (16) and FDA CDER guidance (17). Pharmacopoeia recommends the symmetry factor between 0.8 and 1.5 while CDER guidance below 2.0. According to CDER (17) the minimal values of resolution and number of theoretical plates should be 2.0 and 2000, respectively.

Limit of detection (LOD) and quantitation (LOQ)

Limits of detection (LOD and quantitation (LOQ) were estimated based on the noise threshold and verified experimentally. The theoretically calculated LOD and LOQ as the concentration which is equal to 3 times and 10 times of noise on HPLC 1 are 0.0087 and 0.029 µg/mL, respectively. However, the experimentally verified LOD was a little higher and was established as 0.0125 µg/mL. The relative

Table 1. Mean values of system suitability parameters of TOA signal registered on two columns and two HPLC systems.

System	Column no	Selectivity, α,	Symmetry, A_s	Resolution, R_s	No of theoretical plates, N
HPLC 1	1	1.80	1.46	4.23	2148
HPLC 2	3	1.77	1.10	6.62	5469

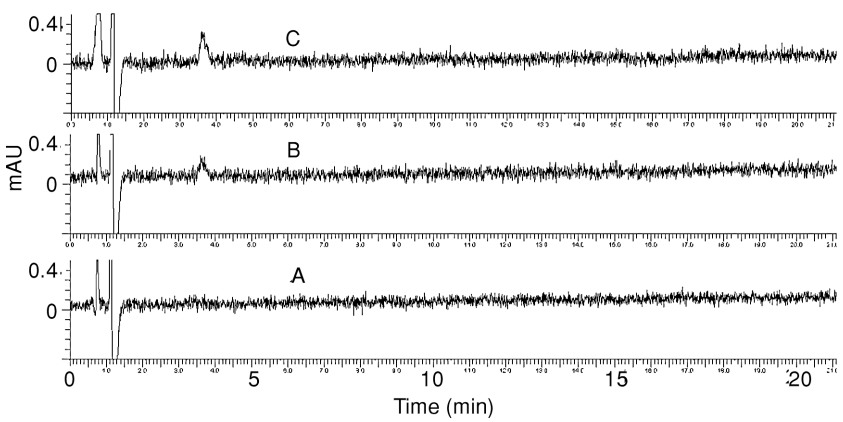

Figure 3. The example chromatograms of: blank – A, TOA (0.0125 µg/mL) – B and TOA (0.0250 µg/mL) – C registered on the HPLC 1 and column 1.

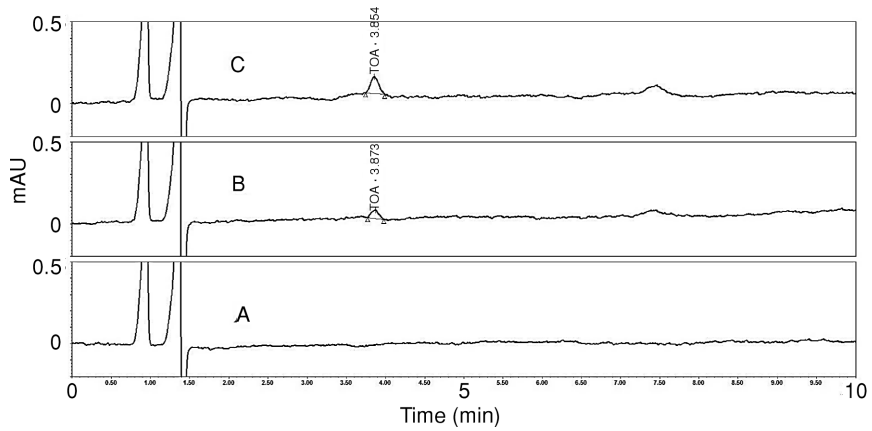

Figure 4. The example chromatograms of: blank – A, TOA (0.005 µg/mL) – B and TOA (0.010 µg/mL) – C, registered on the HPLC 2 by using column 3.

standard deviation of the peak area for these concentrations was 12.4%. ICH guideline (15) defines that: "The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy". Based on this definition as the additional criteria have been established that RSD of peak area, signals of TOA concentration which corresponds to LOQ should be below 10%. The first concentration of TOA which fulfils this requirements (RSD = 8.6%, n = 10) on the HPLC 1 (column 1) is 0.0250 µg/mL. Based on the same assumptions for the HPLC 2 and column 3, the LOD and LOQ were measured as 0.005 µg/mL; and 0.010 µg/mL (RSD = 4.3% , n = 5), respectively. Figures 3 and 4 presents the example chromatograms of blank (buffer) and TOA standards corresponding to LOD and LOQ obtained by using HPLC 1 with column 1 and HPLC 2 with column 3, respectively.

The comparison of LOD and LOQ values obtained by using two different HPLC systems are presented in Table 2.

Linearity

Calibration curve

The linearity of the method was determined by preparing seven standard solutions of TOA within

Table 2. Comparison of limits of detection (LOD) and quantitation (LOQ) obtained by using two HPLC systems and columns.

Equipment	LOD (µg/mL)	LOD (%)	LOQ (µg/mL)	LOQ (%)
HPLC 1	0.0125	0.00125	0.0250	0.0025
HPLC 2	0.005	0.0005	0.010	0.001

Table 3 Statistical analysis of calibration graph for the determination of TOA in DMSA.

Linear range; (µg/mL)	0.125 - 6.00
Number of independent measurements, n	7
Slope, b	123530.4
SD_b,	413.6
Intercept, a	1418.6
SD_a,	1294.5
Mean standard deviation from the regression line	2440.06
Correlation coefficient, R	0.99997
F test value for the regression factor	89212.57
Critical value of $F_{1;\ n-2;\ 0.05}$	7.71

Table 4. Accuracy of determiantion of TOA in DMSA (1 mg/mL).

TOA added (µg/mL)	TOA found (µg/mL) mean value	SD	RSD (%)	Mean recovery in level (%)	Mean recovery $\bar{R}$ (%) Confidence interval (95%)
0	< 0.0125	-	-	-	$\bar{R}$ = 99.1
0.125	0.121	0.0022	1.83	96.6	
0.250	0.243	0.0044	1.82	97.2	SD = 1.8
0.500	0.491	0.0028	0.57	98.3	
1.000	1.000	0.0070	0.70	100.0	ΔR = 0.6
4.000	3.992	0.0194	0.49	99.8	
5.000	5.047	0.0308	0.61	100.9	$\bar{R} \pm \Delta R$ = 99.1 ± 0.6
6.000	6.000	0.0217	0.36	100.0	

the range 0.125-6.00 µg/mL, which corresponds to 0.0125-0.600% with respect to the DMSA concentration. Table 3 presents a statistical analysis of the calibration graph for the determination of TOA in DMSA.

As is clearly evident excellent linearity, confirmed by correlation coefficient and high, compare to critical, the value of F-test for correlation is obtained in the tested range.

Accuracy

Accuracy of the method was demonstrated by the recovery studies, which were carried out by spiking aliquots of DMSA with TOA standards at seven levels of concentrations (4 replicates each). The results are presented in Table 4. The concentrations have been calculated based on the calibration graph equation.

Precision

According to ICH guideline (15) precision should be considered as repeatability (intra assay precision) and intermediate precision which expressed within-laboratories variations: different days, different analysts, different equipment, etc. Repeatability should be assessed using a minimum of 9 determinations covering the specified range for the procedure (e.g. 3 concentrations / 3 replicates each or a minimum of 6 determinations at 100% of the test concentration. To estimate intermediate precision of the method different samples of DMSA spiked with TOA were analyzed for two days, by using different columns (two different batch numbers of Kromasil C18, 100 x 4.6 mm; 5 µm), eluents, and TOA standard solutions. The obtained results are presented in Table 5.

Repeatability can be estimated based on the data presented in Table 4, due to the fact that more than 3 levels (4 replicates each) within the whole range were tested. However, an additional test was done by other analyst for six samples on the HPLC 2 and column 3. The results are presented in Table 6.

The results of the accuracy and precision of the method are on an acceptable level when they are compared with the average values of recovery and relative standard deviation, for similar concentrations of analyte in the sample, presented in the literature (18). According to these literature data in the analysis of a sample containing 0.01%; 0.1% and 1% of an analyte, the average recoveries are in the ranges of 90-107%; 95-105%, and 97-103%, respectively. The average precision of analysis at analyte concentrations 0.01%; 0.1% and 1% are 5.3%, 3.7% and 2.7%, respectively (18).

Robustness of the method

The robustness of the method was tested by changing of flow rate (1.0 ± 0.1 mL/min) composition of acetonitrile (10 ± 1%) and octylamine in the mobile phase. The results of the recovery test (5 replicates) in modified conditions are collected in Table 7. The other factor which can impact the determination of TOA in DMSA is the temperature of the sample. The sample should be kept at a stable temperature. Good results were obtained in the temperature range of 17-22°C. When a sample of DMSA spiked with TOA (1 µg/mL) was put into an

Table 5 Intermediate precision determination of TOA in DMSA. DMSA samples (1 mg/mL) spiked with 5.00 µg/mL of TOA.

Number of measurements, n	Recovery of standard (µg/mL)	Recovery of standard (%)	Mean recovery Comment Mean recovery (%)	$\bar{R}$ (% Confidence interval (95%)
1	5.075	101.5	day 1	
2	4.997	99.9	column 1	
3	5.017	100.3	eluent 1	$\bar{R}$ = 100.0
4	5.003	100.1	(pH = 7.9)	
5	5.004	100.1	TOA solution 1	SD = 0.55
6	4.993	99.9		
7	4.963	99.3		ΔR = 0.35
8	4.989	99.8	day 2	
9	5.011	100.2	column 2	$\bar{R} \pm \Delta R$ = 100.0 ± 0.4
10	4.979	99.6	eluent 2	
11	4.997	99.9	(pH = 8.0)	
12	4.979	99.6	TOA solution 2	

Table 6. Repeatability. Analysis of DMSA (1 mg/mL) samples spiked with TOA (1 µg/mL) standard.

Sample	Recovery of standard					Mean recovery $\bar{R}$ (%) Confidence interval (95%)
	(µg/mL)		(%)			
No	1	2	1	2	mean	
I	1.005	1.010	100.5	101.0	100.7	
II	1.003	1.002	100.3	100.2	100.3	$\bar{R}$ = 100.2
III	1.004	1.001	100.4	100.1	100.2	SD = 0.50
IV	1.004	0.996	100.4	99.6	100.0	RSD = 0.50
V	1.005	0.997	100.5	99.7	100.1	ΔR = 0.3
VI	1.003	0.991	100.3	99.1	99.7	$\bar{R} \pm \Delta R$ = 100.2 ± 0.3

Table 7 Robustness. Influence of changes in standard procedure on the accuracy determination of TOA in DMSA.

Parameter	Flow (mL/min)		ACN (%)		OA (%)	
Value	0.9	1.1	9	11	0.045	0.06
Mean recovery (%)	100.0	101.9	103.6	101.4	97.6	99.6
SD	0.5	0.6	1.4	0.4	1.4	0.3
RSD (%)	0.5	0.6	1.4	0.4	1.4	0.3
Confidence limits (95%), (%)	± 0.5	± 0.6	± 1.6	± 0.4	± 1.5	± 0.3

autosampler without thermosetting, the RSD for the peak area of TOA was above 4%. This is probably due to the fact that the initial temperature of the auto-sampler rack could be even 7°C above room temperature. As mentioned in preliminary results the important factor is also the pH of the sample. Due to the fact that the pH of the TOA standard solution is practically the same as buffer i.e. about 11.0 and the pH of unspiked and spiked DMSA samples is about 8, the question arises if this fact affects the determination of TOA? In this cause a comparison of standard calibration graph (SCG) with a graph obtained by a method of standard addition (MOSA) is useful. MOSA is used in a situation when the difference between the surroundings of analyte in pure standard solution and sample can significantly influence the determination of an analyte (19, 20). The same concentrations of TOA were used to prepare a standard calibration graph (SCG) and MOSA curves. If the matrix effect would be strong, the slopes of the MOSA curve and SCG should be significantly different (19). To verify, whether the difference of slopes of both curves is significant, the t-test was used (21):

$$t_{exp} = \frac{|b_1 - b_2|}{\sqrt{s_{b1}^2 + s_{b2}^2}}$$

where: b_1 – slope of MOSA curve, b_2 – slope of SCG curve, and S_{b1}, S_{b2} are their standard errors. If the calculated value of t_{exp} for f degrees of freedom (f = $n_1 + n_2 - 4$) and probability level 95% is lower than critical, the difference is not significant. The appropriate values for SCG curve are presented in Table 3, while values of slope and their standard deviations for MOSA curve are 124032.8 and 379.5, respectively. The experimental value of t-test value – t_{exp} = 0.895 is lower than critical value – $t_{0.05,50}$ = 2.009, what confirms that both curves are not significantly different.

Stability of solutions

TOA standard solution of 50 µg/mL was stable up to 8 h at temperature 22°C, while solutions of TOA 1 µg/mL kept in the refrigerator were stable even up to four days. The peak area of TOA in spiked samples of DMSA does not change during period of 3.5 h after preparation, however, longer stability was not tested.

Column live-time

Column 1 has been used for the validation process as well as for the analysis of real samples. During all these operations about 320 injections of samples have been made including 90 injections of TOA standards, 158 injections of DMSA (1 mg/mL)

samples spiked with different TOA concentration, 14 injections of unspiked DMSA (1 mg/mL) samples, more than 50 injection of different tested buffers and a few injections of ACDCA and DMSA spiked with TOA and ACDCA samples. On the chromatograms of a few last injections of TOA standard and samples of DMSA spiked with TOA, a small fronting effect with respect to TOA (A_s = 0.89) and DMSA signals was observed. The retention time of TOA signal was decreased slightly (about 1.1%) and still, separation and peak shape were good enough for analysis. However, from this point further degradation of the column was observed – peak fronting increases and retention times of TOA and DMSA made shorter and shorter, and finally, the analysis was not possible. The second column was used for about 300 injections before degradation. The changes in columns performance were observed also on the chromatograms of column test mixture (dimethyl phthalate, toluene, biphenyl, and phenanthrene). The same effects of peaks fronting and decreasing retention times were observed especially in the cause of signals of the last two compounds in the test mixture. All columns were tested according to manufacturer procedure i.e.: mobile phase was a mixture of methanol/water (80/20 v/v); flow rate was 1.0 mL/min and wavelength was 254 nm. Test mixture was composed of: dimethyl phthalate (250 µg/mL); toluene (500 µg/mL), phenanthrene 50 µg/mL and biphenyl (50 µg/mL) in methanol. However, the retention mechanism in the method of TOA determination is different from this in test condition, this column test is an easy tool to check if the column is still suitable for analysis of TOA using the ion-pair mobile phase. There is no satisfactory explanation of peak fronting in this situation. In literature (22) as one of the possible sources of peak fronting is suggested using the mobile phase outside the pH range of stability of the column. According to the manufacturer of all columns used in these experiments, the recommended mobile phase pH is between 1.5 and 9.5. The pH of mobile phases that have been used in experiments and analysis was usually between 7.8 and 8.0 and only in one case was 8.3. The second hypothesis was possible contamination of stationary phase by metal ions washed out from metal elements of HPLC as well as from reagents used for mobile phase preparation and its possible reaction with TOA and DMSA during analysis. Indeed, in concentrated ortho-phosphoric acid and octylamine used for the preparation of the mobile phase quite high amount of metal ions was determined i.e. about 13.2 ppm of Al, 4.0 ppm of Fe, 54.1 ppm of Ca, and 19.6 ppm of Mg in H_3PO_4 and even 93.6 ppm of Zn in octylamine. However, in the mobile phase before column conditioning, it was found only 0.46 ppm of Al. Moreover, in the mobile phase after column conditioning, the amount of Al was practically the same (0.45 ppm). In analyzed samples of the stationary phase, it was determined 2.8 ppm of Al, 4.1 ppm of B, 1.3 ppm of Ca, 1 ppm of Fe, and 1.3 ppm of Mg. In the certificate of analysis silica gel used for the preparation of this column, it was declared below 1 ppm of Al and 2 ppm of Fe. So, the determined level of aluminum was only slightly higher and iron was even below the level declared by the manufacturer. Some other experiments suggest that degradation of the stationary phase occurs at a small layer near the column inlet. Maybe hydrophobic interaction (RP mechanism), which probably coexist with typical ion-pair interaction, caused slow accommodation of probe and as result – TOA peak fronting. On the other hand, there was no observed TOA peak fronting and changing of column pressure during column live-time.

CONCLUSION

The developed method of determination of TOA in DMSA is selective, sensitive, accurate (mean recovery of standard > 99%) and precise (RSD in the range of 0.4-1.8%, depends on concentration) enough for analysis of thioacetic acid content in DMSA samples. Some problem is not satisfactory column life-time.

Conflict of interest

The authors declare no conflict of interest.

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