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ANALYSIS

SIMULTANEOUS DETERMINATION OF ETHYLENEDIAMINE-N,N,N',N'-TETRAKISMETHYLENE PHOSPHONATE AND ASCORBIC ACID IN „COLD" RADIOPHARMACEUTICAL KIT

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Abstract: Ethylenediamine-N,N,N',N'-tetrakismethylene phosphonate (EDTMP) and ascorbic acid (AA) were determined in two batches of lyophilized experimental radiopharmaceutical kits by using ion chromatography with UV detection. The separation of EDTM and AA was carried out on PRP-X 800 weak cation exchange column and as an eluant 0.0025 mol L^{-1} sodium sulfate was used. Validation parameters of both analytical procedures: limit of detection (LOD), limit of quantitation (LOQ), linearity, precision, accuracy and system suitability test (SST) parameters are presented.

Keywords: ethylenediamine-N,N,N',N'-tetrakismethylene phosphonate and ascorbic acid determination, validation of analytical procedures, standard calibration graph, method of standard addition

Ethylenediamine-N,N,N',N'-tetrakismethylene phosphonate (EDTMP) is a ligand widely used in radiotherapy. Its complexes with ions of radioactive elements, e.g. ^{153}Sm(III) or ^{188}Re (IV/V) are carriers of therapeutic doses of ionizing radiation to specific disease sites with specificity in the body (1). ^{153}Sm-EDTMP exhibits special affinity for bone and concentrates in areas of bone turnover in association with hydroxyapatite. It is widely used in therapy of skeletal metastases, metastatic cancer of bone (1, 2), multiple myeloma (3). Labelled ^{188}Re EDTMP has been proposed for bone pain palliation (4). Chemical properties, pharmacokinetics and HPLC separation of ^{153}Sm-EDTMP and ^{186}Re-EDTMP complexes were described in the literature (5). Recently, potential Ga(III)-EDTMP antitumor effect has been investigated (6). Ion exchange separation of EDTMP and other similar chelators (NTA, EDTA, CDTA, DTPA, EDDS, EDTA derivatives HEDTA, ED3A) have been reported a few years ago (7). Ascorbic acid (AA) is often used in pharmacy excipients and a lot of official (8) and non official literature (9-11) reporting its determination in pharmaceuticals have been published to this time. Official, pharmacopeial method of EDTMP determination in radiopharmaceutical kits has not been published, yet.

The aim of this work was to develop a method of separation and simultaneous determination of EDTMP and AA in samples of radiopharmaceutical experimental kit, to validate elaborated procedures and finally to check if storage conditions change them quantitatively.

EXPERIMENTAL

Apparatus

The chromatographic system was composed of the following instruments: a binary LC 210 ProStar HPLC pump (Varian, USA), a Rheodyne Model 7125 injection valve equipped with a 20 mL sample loop (Cotati, CA, USA), 345 dual mode UV/VIS detector (Varian). The chromatographic separation was carried out on the: PRP-X-800 (Hamilton): 250 × 4.6 mm, 7 mm, weak cation exchange column. The acquisition and handling of the data were carried out with a STAR 6.2 software (Varian). The UV spectra were registered on SP-800 UV-VIS spectrophotometer (Unicam, UK). For pH measurements a CP-401 (Elmetron, Poland) pH meter with combined electrode was used.

Materials and Reagents

All of the reagents used were of analytical reagent grade. EDTMP was synthesized in Mannich

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type reaction according to (12) and checked by ^{1}H NMR and ^{31}P NMR (5, 12).

EDTMP stock solution (0.5 mg mL^{-1}) was prepared by pouring 0.1000 g pure substance to 200 mL calibrated flask and dissolving it in water with shaking. The dissolution proceeded quite slowly.

The stock solution of L-(+)-ascorbic acid (Merck) was prepared every 4 h. Concentrated stock solution (1 mg mL^{-1}) was obtained by dissolution of 0.1000 g AA in 100 mL of distilled water. Stock solution used for SCG preparation and sample spiking (0.1 mg mL^{-1}, pH 7.5-7.7) was obtained by dilution (1:10) of concentrated stock solution, and the pH was adjusted by 0.1 M sodium hydroxide.

Sample

The kit vials of experimental series 01/05 and 03/06 produced by POLATOM contains about 10 mg of EDTMP, 2 mg of ascorbic acid, 0.2 mg of tin(II) chloride and some other excipients e.g. NaOH (necessary to adjust the pH of the water solution to about 7.5). The total mass of the vial content was about 20 mg.

Sample stock solutions were prepared by dissolution of the vials content in distilled water and filling up with water to 50 mL. The pH of the solutions was about 7.5.

Preparation of solutions for calibration graphs

Solutions of ascorbic acid (concentrations range 2.5 – 40.0 µg mL^{-1}) were prepared by appropriate dilution of 0.1 mg mL^{-1} stock AA solution. Solutions of EDTMP (concentrations: 25.0 – 500.0 µg mL^{-1}) for standard calibration graph (SCG) construction were prepared by appropriate dilution of EDTMP stock solution. EDTMP solutions for preparation of the method of standard addition (MOSA) curve were obtained as follows: to 10 mL volumetric flasks were poured 5 mL of sample stock solution and appropriate volumes of stock EDTMP solution – depending on desired EDTMP standard concentration in spiked samples. The volumetric flask were filled up to the mark with distilled water.

Procedure

Unspiked samples

To 10 mL volumetric flask 5 mL of stock sample solution was transferred and its content was filled up to the mark with distilled water. 20 µL of the sample was injected on the column and analyzed with detection at 205 and 254 nm.

Samples spiked with EDTMP standard (for recovery determination)

To 10 mL volumetric flask 5 mL of sample stock solution and 0.5 mL (50 mg mL^{-1}) or 1 mL (100 mg mL^{-1}) stock solutions of EDTMP were added and their contents were filled up to the mark with water. 20 µL of the sample was injected on the column and analyzed with detection at 205 and 254 nm.

Samples spiked with AA

To 10 mL calibrated flask 5 mL of stock sample solution and 1 mL AA solution (0.1 mg mL^{-1}) were transferred and its content was filled up to the mark with distilled water. 20 µL of the sample was injected on the column and analyzed with detection at 254 nm.

Eluent

As an eluent 0.0025 mol L^{-1} sodium sulfate was used. Before use the eluent was subjected to sonication for 15 min.

RESULTS AND DISCUSSION

Preliminary spectrophotometric experiments

In previous work it was found that AA water solutions at pH between 6.0 and 8.5 are stable for about 4 h (13). Stock solutions of AA were prepared with this frequency.

EDTMP exhibits weak absorbance in the short UV region. Due to interferences from ascorbic acid and tin(II) chloride present in the kit, direct spectrophotomeric determination of EDTMP was found to be improper. The influence of pH on the EDTMP water solutions absorbance was tested. The pH of the solutions (except values 3.2 observed for pure 0.5 mg mL^{-1} EDTMP) was adjusted by diluted hydrochloric acid or sodium hydroxide solutions. It was found that the measured absorbance values are constant over the wide pH range (4.0 – 8.0).

Preliminary chromatographic experiments

In preliminary HPLC experiments various Microsorb reverse phase columns (C_{18} and C_8 – both phases 4.6 × 250 mm, 100 Å) and ion exchange ones (PRP-X 800 – cation exchange, AN-1- anion exchange) were tested. Separation of AA and EDTMP signals on reverse phase columns was not satisfactory (the resolution was poor) even when a weak eluent (water) was used. However, good separation of both substances was obtained on PRP-X 800 weak cation-exchange phase with 0.0025 mol L^{-1} sodium sulfate solution as an eluent. The flow rate was 0.8 mL min^{-1}. Ascorbic acid and EDTMP

absorb at different wavelengths and EDTMP band is weak at the maximum absorption of AA. Therefore detection was carried out simultaneously at two wavelengths: 205 nm for EDTMP and 254 nm for AA determinations (Fig.1).

In contrast to spectrophotometric study it has been found that the pH of the EDTMP samples between 3.2 and 7.0 does not influence on quantitative analysis. The peak areas of EDTMP signals for standard solutions (250 µg mL^{-1}) at pH 3.2 and 7.0 are practically the same. This can be explained by significant dilution of small volume of EDTMP solution with the eluent. During chromatographic analysis 20 µL of injected sample is diluted by the volume of eluent in the column. Such dilution results in a final pH of the obtained solution about 5.3 which is within the optimal range of pH for spectrophotometric analysis. The analysis of the examined samples is short (5 min), as after second signal (AA) no additional signals are registered during 30 min.

Validation of developed methods

System suitability test

EDTMP determination

Figure 2 shows typical chromatograms of kit samples prepared according to Procedure – unspiked and spiked with EDTMP.

EDTMP peak is well separated from neighboring one (AA) and symmetrical; its symmetry factor calculated as:

$$T = W/2W_a \quad [1]$$

(where W is a peak width at 10% height from the baseline and W_a is a peak front edge width at the same height) changes within a range 1.02 to 1.19 (mean value from 20 replicates = 1.15). The European Pharmacopoeia in general requirements state that *"the symmetry factor of the principal peak is to be between 0.8 and 1.5 unless otherwise stated in the monograph."* (14). Separation of signals was

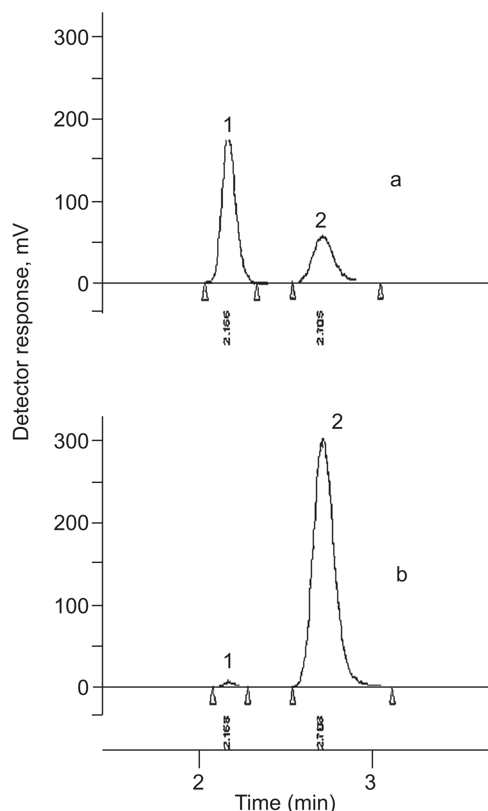

Figure 1. Chromatograms of the mixtures of AA and EDTMP (CAA = 20 µg mL-1, CEDTMP = 100 µg mL-1): a - registered at 205 nm (EDTMP determination), b - registered at 254 nm (AA determination); 1. - EDTMP, 2 - AA.

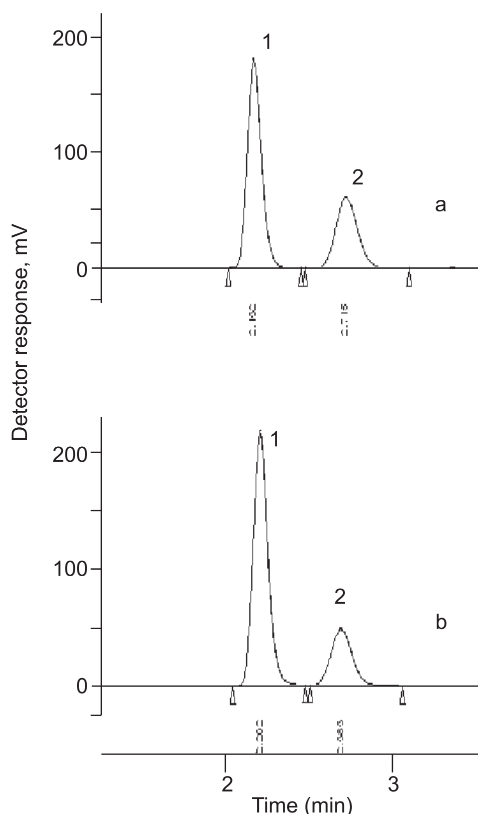

Figure 2. EDTMP determination (205 nm). a - chromatogram of unspiked sample; b - chromatogram of the sample spiked with 250 µg of EDTMP standard; 1 - EDTMP, 2 - AA

quantitatively described by resolution calculated in usual way (14, 15). In presented chromatographic procedure resolution (R_s) equals about 2.5. A resolution greater than 1.5 corresponds to baseline separation (14, 15). The values of symmetry factor and resolution fulfil the requirements and recommendation of the European Pharmacopoeia. The efficiency of separation was estimated based on the number of theoretical plates per meter (14, 15) and ranged from 10934 m^{-1} to 12241 m^{-1} (mean value 11702 m^{-1}).

Regression lines. Accuracy and precision

Chromatographic determination of EDTMP was performed using standard calibration graph (SCG) and method of standard addition (MOSA). In MOSA procedure sample is spiked by the standard of analyzed substance and therefore is in the same surroundings as the analyte. The same procedural operations are performed on both, spiked and unspiked sample and constant proportional bias is introduced onto sample across dynamic range (14, 16, 17). The comparison of these two methods (SCG and MOSA) can give information about evidence of the matrix (in this case other components of the kit) effect. If slopes of the standard calibration graph and MOSA are significantly different, the suggestion is that matrix effect is strong and determination based on SCG is not accurate (18). For comparison of the discussed slopes, t-test was used (equation 2)

$$t_{exp} = |b_1 - b_2| \Big/ \sqrt{s_{b1}^2 + s_{b2}^2} \qquad [2]$$

where b_1 is a slope of MOSA curves (for 01/05 or 03/05 series) and b_2 is a slope of standard calibration graph; whereas s_{b1} and s_{b2} are standard deviations of b_1 and b_2, respectively. The calculated t_{exp}value for 01/05 MOSA-SCG curves is 0.525. For 03/06 MOSA-SCG curves this value is 0.260. Both of them exclude the appearance of matrix effect on EDTMP determination – theoretical values $t_{0.05, N1+N2-4}$ = 1.796 >> $t_{calculated}$ (N = number of concentrations multiplied by number of replicates). Statistical evaluation of the obtained standard calibration graph curves and MOSA (for both analyzed samples of radiopharmaceutical kit) are presented in Table 1.

The data from Table 1 prove good linearity and correlation of calibration curves. Both MOSA curves and SCG are linear in EDTMP concentration range (up to 250 µg mL^{-1} for MOSA and 25 – 500 mg mL^{-1} for SCG) and their correlation coefficients (r) exceed 0.999. Although there is not accepted the lowest correlation coefficient value, even in analysis of pharmaceuticals – e.g. Tønseth and Døhl (19) propose r > 0.985 – the presented methods fulfil even the most rigorous requirements (r = 0.999) recommended in the literature (20). The significance of the correlation was tested by the F-test and the obtained results indicated good fitness ($F_{experimental}$

Table 1. Comparison of standard calibration graph (SCG) and method of standard addition (MOSA) for EDTMP determination.

	SCG	MOSA	
Batches		01/05	03/06
Detection limit (µg mL^{-1})[a]	6.8		
Quantitation limit (µg mL^{-1})[b]	22.7		
Linear range (µg mL^{-1})	25.0 – 500.0	0.0 – 250.0	0.0 – 250.0
Number of independent measurements, n	7	5	5
Slope, a	10920	11006	10975
RSD_a (%)[c]	0.49	1.41	1.86
Intercept, b	17615	1081526	1131137
RSD_b (%)[c]	70.98	1.99	2.51
SD[d]	24784	34960	46067
Correlation coefficient, r	0.99994	0.9996	0.9993
F-test for correlation	13919	5032	2867
$F_{1; 4; 0.05}$	6.61	7.71	7.71

[a]Detection limit was calculated based on calibration graph as LOD = 3 × SD/a**,** where SD is the mean standard deviation of regression, and a is a slope of calibration graph. [b] Quantitation limit was calculated, in similar way, as LOQ = 10 × SD/a. [c] RSD_a and RSD_b are relative standard deviations of a and b, respectively. [d] SD – the mean standard deviation of regression.

Table 2. Comparison of precision and accuracy for EDTMP determination by SCG and MOSA methods in two batches.

Batch	Method	Added (μg mL^{-1})	Found (μg mL^{-1})	RSD (%) (n = 3)	Recovery (%)	Content, mg per vial (significance level 95%)
01/05	SCG	-	98.6	1.20	-	9.86 ± 0.35
		50.0	50.7	2.6	96.7	
		100.0	99.9	0.70	104.4	
	MOSA	-	98.8	3.19	-	9.88 ± 0.67
		50.0	48.2	1.75	96.4	
		100.0	98.3	1.25	98.3	
03/06	SCG	-	103.2	1.70	-	10.32 ± 0.52
		50.0	51.2	1.37	99.2	
		100.0	100.7	1.47	101.6	
	MOSA	-	103.1	4.10	-	10.31 ± 0.90
		50.0	54.4	1.36	103.9	
		100.0	104.1	1.19	104.1	

>> $F_{theoretical}$). When MOSA was used, concentration of EDTMP in analyzed samples, x_s, was obtained by extrapolation of the standard addition curve up to y = 0 (where y is the measured analytical signal – peak area) or calculated as $x_s = a/b$ (where a and b are the coefficients from regression line). Relative standard deviations for the results obtained by MOSA method were calculated (14, 16, 17) as $RSD(x_s) = S_{xs}/x_s \times 100\%$, where:

$$S_{xs} = \frac{SD}{a}\sqrt{\frac{1}{n} + \frac{\bar{Y}^2}{a^2\sum_{1}^{n}(X-\bar{X})^2}} \quad [3]$$

(SD – mean standard deviation of regression, n – number of spiked samples prepared for the construction of the standard addition curve, a – slope of the standard addition curve, $\bar{Y}$ – mean value of measured analytical signal, X – concentration of added EDTMP, $\bar{X}$ – mean value of added EDTMP concentrations.)

Both methods are precise and accurate. The accuracy was estimated based on recovery of standard as $c_{measured}/c_{added} \times 100\%$ (14). The recovery, depending on the added EDTMP amount (Tab. 2), varies for both batches from 96.7 % to 104.4 % and between 96.4 to 103.9 when calculated based on SCG and MOSA, respectively.

The results of the EDTMP determination in both samples, calculated from standard calibration

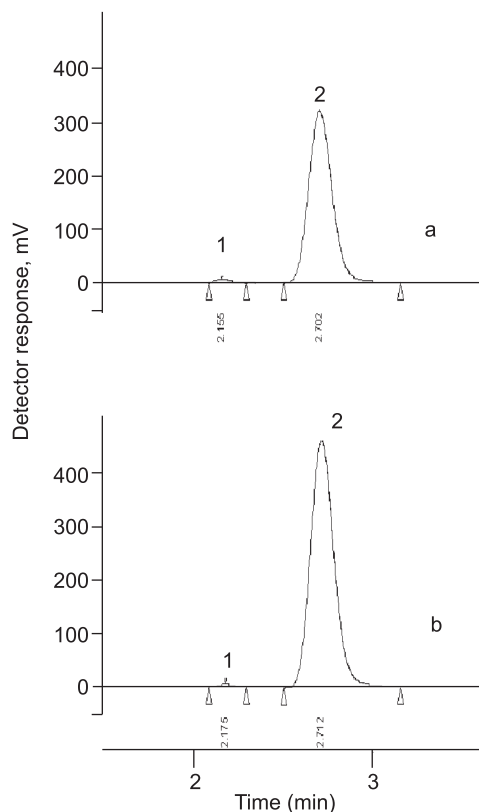

Figure 3. AA determination (chromatograms of samples registered at 254 nm) a - chromatogram of unspiked sample, b - chromatogram of the sample spiked with AA (100 g); 1 - EDTMP, 2

Table 3. System suitability test parameters for ascorbic acid determination

Parameter	Mean value (range)
Retention time (min)	2.69 (2.64 – 2.71)
Retention factor	0.50 (0.46 – 0.51)
Number of theoretical plates/m	8416 (7978 – 9426)
Symmetry factor, T	1.14 (1.11 – 1.15)
Resolution, R	2.5 (2.3 – 2.7)
Selectivity factor, $\alpha_{AA/EDTMP}$	2.35 (2.18 – 2.52)

Table 4. Detection, quantification limits and calibration graph for ascorbic acid determination (at 254 nm)

Detection limit (μg mL^{-1})	0.10
Quantitation limit (μg mL^{-1})	0.35
Linear range (μg mL^{-1})	2.5 – 40.0
Number of independent measurements (n)	5
Slope, *a*	123990
RSD_a (%)	0.11
Intercept, *b*	27405
RSD_b (%)	10.6
SD	4305
Correlation coefficient, *r*	0.999998
F-test for correlation	771363

Abbreviations, symbols and footnotes used in this Table are the same as in Table 1.

graph (Tab. 2) are in good agreement with MOSA ones. However, analysis based on standard calibration graph is less laborious, more precise and accurate than MOSA (cf. Table 2), therefore it is recommended for EDTMP determination.

Validation of the method for AA determination

Typical chromatograms of samples: alone and spiked with AA are presented in Fig. 3

All parameters which are commonly named as system suitability test (symmetry factor, number of theoretical plates, resolution) were calculated in the same way as for EDTMP. The selectivity (separation) factor was taken as: $a_1 = k_{AA}/k_{EDTMP}$, where k_{AA} and k_{EDTMP} are retention factors for AA and EDTMP, respectively. System suitability parameters are presented in Table 3.

The value of selectivity (separation) factor – is recommended above 1.05 to obtain minimal acceptable separation (15) – and resolution confirms good selectivity and separation of the developed method.

Regression lines, accuracy

The limits of detection and quantitation were calculated in the same way as in case of EDTMP. Regression line equation (SCG curve); LOD and LOQ values for AA determination are presented in Table 4. Accuracy and precision are presented in Table 5.

The results presented in Table 4 confirm good correlation (high F-test value, and *r* values close to 1) and high sensitivity of the method. The slope of the AA curve is about 10 times greater and limit of detection is over 60 times lower in comparison to slope of SCG and LOD for EDTMP determination at 205 nm.

The accuracy for both batches varies between 101.3% and 102.0%. The precision of AA determination measured by relative standard deviation (RSD) is similar for this observed for EDTMP determination and varies between 0.6% and 4.2 %.

CONCLUSIONS

The chromatographic procedure described is simple (isocratic elution and UV detection), fast and can be carried out on every HPLC apparatus, commonly used in most popular RP-HPLC. The method developed allows for good separation and rapid determination of ascorbic acid and EDTMP in tested radiopharmaceutical kit. The procedure fulfils most requirements specified for pharmaceutical

Table 5. Accuracy and precision determination of ascorbic acid in the sample kit

Batch 01/05				
Added ($\mu g\ mL^{-1}$)	Found ($\mu g\ mL^{-1}$)	RSD, % (n = 3)	Recovery (%)	AA concentration (mg per vial) (95% significance level)
-	21.9	4.2	-	2.19 ± 0.21
10.0	10.2	2.5	102.0	
Batch 03/06				
Added ($\mu g\ mL^{-1}$)	Found, ($\mu g\ mL^{-1}$)	RSD (%) (n = 3)	Recovery (%)	AA concentration (mg per vial) (95% significance level)
-	22.0	2.5	-	2.20 ± 0.19
10.0	10.1	0.60	101.3	

analysis. The concentrations of EDTMP and AA in both samples (batch 01/05 and 03/06) are within required range. Storage in a fridge for about one year (batch 01/05 and 03/06 have been prepared within 13 month interval) does not influence meaningfully on the EDTMP and AA content in kit vials.

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