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Simultaneous Determination of Thimerosal and Aluminum in Vaccines and Pharmaceuticals with the Use of HPLC Method

M. ZARĘBA*, P.T. SANECKI, AND R. RAWSKI

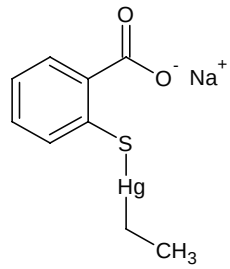
Department of General Chemistry and Electrochemistry, Faculty of Chemistry,
Rzeszow University of Technology, 35-959 Rzeszow, Poland
*E-mail: magzar@prz.edu.pl

Summary. A simple and convenient chromatographic method of simultaneous separation, identification, and quantitative determination of thimerosal (TM) (preservative) and aluminum (adjuvant) in vaccines and pharmaceuticals by reversed phase high-performance liquid chromatography (RP-HPLC) with visible (VIS) detection was developed and validated. Due to postcolumn derivatization with dithizone, any interference from matrix was excluded. Similarly, a possibility of on-column decomposition of dithizonates was eliminated. Evaluated detection limits were 0.3 μg TM and 3.0 μg Al, which correspond to the smallest, but possible to recognize, visible peak.

Key Words: thimerosal, aluminum, HPLC, dithizone, vaccines

Introduction

Thimerosal [1] (thimerosal, merthiolate) is the sodium salt of ethylmercurythiosalicylic acid [($ArCO_2$)SHgEt]Na. It was introduced to the market in 1920 [2] as antibacterial and antifungal agent and is still being used as an antiseptic and preservative in various formulations [3–5].



The degradation of thimerosal (TM) involves creation of ethylmercury cation ($CH_3CH_2Hg^+$) [6–10]. Further metabolism of $EtHg^+$ intermediate in-

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cludes stage of dealkylation to inorganic form of mercury Hg^{2+} ion [11, 12], which can be followed by its methylation to methylmercury form with B12 participation [13–15]. Mercury and its organic and inorganic forms are well recognized as the highly toxic substances [16, 17] for which the primary target is the central nervous system [18–21]. Therefore, the determination of its level in vaccines is still valid. In the present paper, we describe a convenient method of simultaneous determination of TM and another important component of vaccine, namely, Al compounds. The latter is routinely used in human vaccines as adjuvant. It induces an early, efficient, and long-lasting protective immunity. Its amount must be controlled due to biotoxicity of Al. Usually, less than 1 mg Al is applied per one vaccine dose. Aluminum phosphate or aluminum hydroxide is, in most cases, used [22–25].

Determination of thimerosal in pharmaceuticals is carried out in two ways: as total Hg [26–34] or directly as thimerosal [35–44]. The total Hg determination methods require mineralization and atomization of sample to form mercury vapor. However, there are also a few methods where there is no need to apply volatile mercury generation since intact TM is determined. Examples include high-performance liquid chromatography (HPLC) [7, 10, 35–40] and a few electrochemical methods [42] as well as ultraviolet–visible (UV–vis) spectrometry [43, 44]. HPLC determination of TM in pharmaceuticals can be realized with spectrophotometric [7, 8, 36–38], electrochemical [39, 40], or inductively coupled plasma mass spectrometry detection [45, 46]. The respective spectrophotometric detection is typically realized in UV range [7, 36–38, 40].

Colorimetric method for mercury analysis with the use of dithizone (DT) as complexion agent is one of the earliest methods for mercury determination [47–50]. Nevertheless, evaluation of metal containing species in visible (VIS) range requires derivatization stage with a suitable chromophore. Dithizone [51] or dithiocarbamate [8, 10] is used for mercury analysis. Dithizone [52] is utilized for speciation of mercury compounds due to its ability to form stable chelates with mercury containing compounds in the ratio of 1:2 or 1:1 with inorganic (Hg^{2+}) and organomercury cation (RHg^{+}), respectively [53]. In HPLC method, the off- or on-column derivatization is applied. In HPLC determination of TM, formation of chelates with DT is carried out as precolumn derivatization. However, in such a method, an interference of present species can occur due to partial on-column decomposition of dithizonates [51, 54]. Taking into consideration these disadvantages, in this paper, the process of derivatization was positioned after separation of components with the use of dithizone as chromophore. Besides, the use

of VIS range detection makes it possible to determine both TM and Al in the same sample simultaneously, since the latter is also considered as biologically toxic and therefore important component.

No need for sample pretreatment is the advantage of the reversed phase high-performance liquid chromatography (RP-HPLC) method proposed here for TM and Al determination. Thimerosal and aluminum present in vaccines is directly detected after chromatographic separation and postcolumn coloring with dithizone. Moreover, postcolumn derivatization allows to avoid a possibility of decomposition of dithizonates on column. As a result, modified free mobile phase is used (e.g., EDTA to complex decomposition products), which is necessary when organomercury compound is separated as dithizonate in precolumn manner.

In conclusion, the presented method provides a fast, simple, and accurate method for control of pharmaceuticals and similar products containing thimerosal and aluminum jointly or individually.

Experimental Part

Reagents

All reagents, i.e., Thimerosal BioXtra (Sigma, USA), aluminum chloride (Sigma, USA), Dithizone (POCH, Poland), and NaOH pellets (Honeywell, Germany) were of analytical grade and used without further purification. Acetone (POCH, Poland), methanol (POCH, Poland), and acetic acid (Sigma, USA) were of HPLC grade. The DTP vaccine 0.5 mL vial (Biomed, Poland) was stored in the refrigerator at 4 °C.

Solutions

The TM stock solution of 150 μg mL^{-1} and its standards at concentrations 30, 50, 70, 90, 110, and 130 μg mL^{-1} in high-purity water were prepared fresh daily and protected from the light. Aluminum stock solution of 1000 μg mL^{-1} in water with addition of hydrochloric acid to avoid hydrolysis was prepared 1 day before. Aluminum standards at concentrations 500, 550, 600, 650, 700, 750, 800, 850, 900, and 950 μg mL^{-1} were done with respective dilution of aluminum stock solution. Dithizone (DT) of 20 μg mL^{-1} solution in acetone was stored in the refrigerator. All standard solutions

were filtered through 0.22 μm filter before injection. Complexing solution according to Shrivastaw and Singh [43] was prepared daily by mixing in the order: water, 20 μg mL^{-1} DT in acetone, acetone, and 12.5 M NaOH in 9:10:10:1 volume ratio.

Chromatography

The HPLC (HITACHI, model LaChrom ULTRA VWR) system equipped with L-2200U Autosampler, L2160U Pump, and UV–Vis Diode Array Detector L-2455U were used.

A LiChroCART(R) stainless-steel column (125 × 4 mm, 5 μm LiChrospher(R) 100 RP-18; Merck) was used. The mobile phase (pH = 3.8) was methanol–water mixture (3:2, v/v) containing 0.02 M acetic acid [39]. Eluent was purified with the use of Millipore purification system with 0.22-μm pore size membrane filter and degassed by means of ultrasound and vacuum. The pressure at flow rate of 1.2 mL min^{-1} in isocratic condition was 74 bar. The oven temperature was set on 30 °C. Analytical time required for analysis of 30 μL injection volume was 10 min. Mobile phase and complexing solution were mixed in the breeder at volume ratio of 3:7. VIS detection for TM at 538 and for Al at 557 nm with the use of diode array detector was performed with retention time of 2.91 min and 7.52 min for Al and TM, respectively.

Spectrophotometry

Spectrophotometer U-5100 Hitachi was used to determine wavelengths appropriate for analytical purpose. Recording of TM–DT and Al–DT complexes spectra was carried out according to the procedure in literature [43] with the use of stock solutions of both species.

Vaccine Analysis

Lowering of pH uniforms all forms of Al potentially present in vaccines ($Al(OH)_3$, Al_2O_3, phosphate, sulphate), into Al^{3+} cation. Therefore, the DTP vaccine (Biomed, Poland) was treated with diluted HCl and filtered through 0.22 μm filter and 30 μL of sample volume was injected. Each experiment was repeated five times, and the results were averaged.

Results and Discussion

The Problem of TM and Al Simultaneous Determination

The use of DT as complexing agent is a well-known method of TM determination [43, 51, 54, 55]. Standard spectrophotometric method, recommended by World Health Organization (WHO) [55], requires a pretreatment stage of TM extraction from water solution to chloroform–DT phase. Another method with DT application does not require extraction stage; to form the Hg–DT complex, the complexing solution containing respective volumes of water, acetone, and 20 μg mL^{-1} DT is used and the appropriate pH value is obtained by adding 12.5 M NaOH [43]. The weak point of the method is nonselectivity of DT [56]. Therefore, if other metals are present, the method gives overstated concentration results. It was confirmed by the simple test with the use of the complexing solution: overstated content of TM in DTP vaccine due to presence of Al was obtained. Therefore, if DT as complexing agent is applied, earlier resolution of TM and Al is necessary. Moreover, as a method of chelates formation, postcolumn derivatization was applied to avoid metal–DT complex and DT alone decomposition on column. It is worth to mention that precolumn derivatization is much more complicated: it requires extraction to chloroform–DT phase [49, 55] and addition of modifier to mobile phase, e.g., EDTA [51] due to partial decomposition of dithizonates. Moreover, precolumn derivatization leads to the presence of many species and peaks and their interference [51, 54].

As mobile phase, a mixture of methanol, water, and acetic acid was used since the latter stabilizes the TM–DT complex for at least 1 h [49]. Fortunately, the same system turned out to be suitable for Al ion determination. The final pH value was optimized in relation to TM–DT complex retention time and column specification. In case of Al, pH changes practically do not influence its complex retention time.

Postcolumn derivatization requires the proper content of complexing solution which should be high enough to obtain stable complexes. Therefore, experimental optimization of mobile phase and complexing solution ratio was required. Optimum value for chromatographic resolution was 70% of complexing solution and 30% (*v*/*v*) of mobile phase at flow rate of 1 mL min^{-1}. It results in retention time of TM and Al complexes below 10 min *(Fig. 1)*.

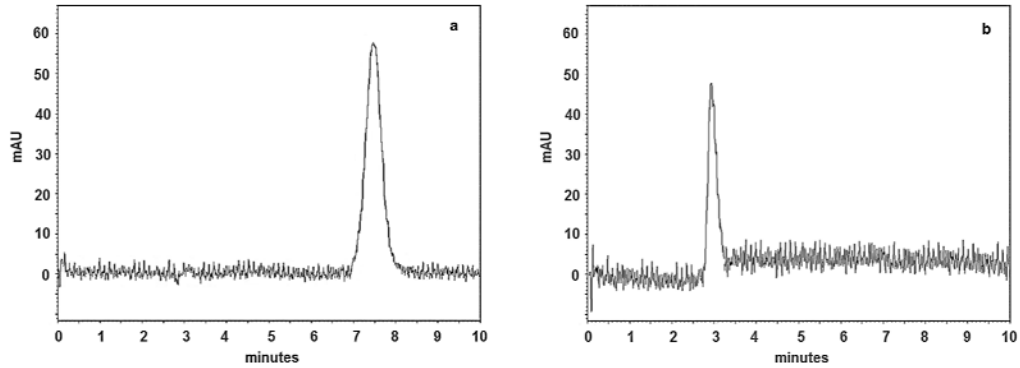

Fig. 1. Chromatograms of TM–DT (a) (R_t = 7.52 min) and Al–DT (b) (R_t = 2.91min) complexes, recorded separately with the use of original standards. mAU — milliabsorbance units

The peak areas and peak high were measured for quantitative analysis. Analytical wavelength of TM–DT and Al–DT complexes was 538 and 557 nm, respectively *(Fig. 2)*.

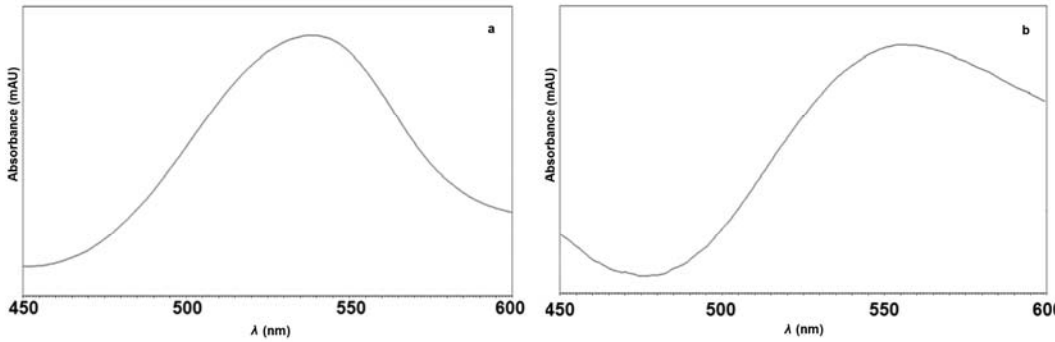

Fig. 2. VIS spectrum of TM–DT (a) (λ_{max} = 538 nm) and Al^{3+}–DT (b) (λ_{max} = 557 nm) complexes for stock solutions of both species and complexing solution of DT

A question arises as to what is the structure of a complex forming in DT–TM reaction. There is no data available concerning directly DT–TM reaction product. For analytical aim, it is of no importance; it is satisfactory that such a relative stable complex exists and is suitable for TM determination [43]. On the other hand, literature data clearly indicate that cation RHg^+ reacts with DT to form chelates with 1:1 ratio [51, 54]. TM, as the compound with polarized S–Hg bond, C_2H_5–$Hg^{\delta+}$–$^{\delta-}$S–C_6H_4–COO^- Na^+, undergoes

cleavage in solution induced by, e.g., light or temperature [7, 10, 36–39]. Consequently, thiosalicylate anion is labile [8] and can be exchanged by stronger nucleophile, e.g., DT ligand. Moreover, with DT, N-S chelate ring can be formed [52, 56], which stabilizes the final complex. Therefore, it is very probable that the respective complex is the ethylmercury–DT one [8].

In the present paper, DT was applied as a postcolumn chelator also for Al^{3+}. Fortunately, it turned out that DT is good for simultaneous determination of TM and Al and there was no need to apply another chelator even if application of other chelators for precolumn determination of Al^{3+} as 8-quinolinol [57], morin [58], and lumogallion [59] is described in literature. The colorimetric determination of Al with the use of DT is not explicitly described in literature. Therefore, some results concerning Al–DT complex are necessary. In our case, Al–DT complex with absorption maximum λ_{max} = 557 ± 3 nm is forming in postcolumn derivatization where Al remains in fully soluble form. For comparison, λ_{max} for DT alone is 478 nm [43], and in this paper, 480 ± 3 nm; for $Hg(DT)_2$, complex is 490 nm [51]; for TM–DT, complex is 538 nm [43].

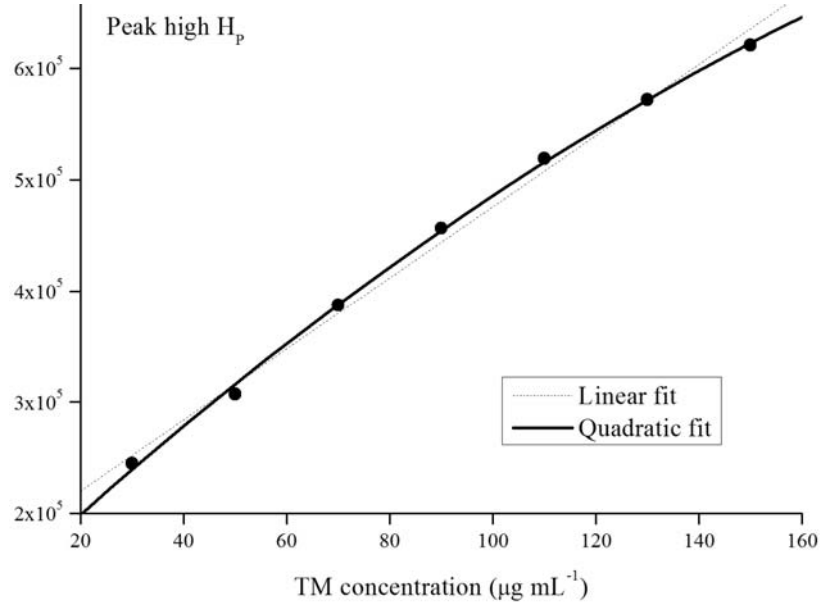

Fig. 3. Calibration plot: peak high Hp vs. TM concentration. Linear regression equations $Y = A + B \cdot X$: $H_p = (1.563 \cdot 10^5) + (0.03196 \cdot 10^5) \cdot C_{TM}$, $r = 0.9970$, $sd_A = 0.1096 \cdot 10^5$; $sd_B = 0.001110 \cdot 10^5$. Quadratic regression equation: $Y = A + B_1 \cdot X + B_2 \cdot X^2$, $H_p = 1.14275 \cdot 10^5 + 4.361 \cdot 10^3\ x - 6.471\ x^2$; $sd_A = 1.142\ 10^4$; $sd_{B1} = 285.1$, $sd_{B2} = 1.555$; $R = 0.9994$.The results obtained from peak area are almost identical

According to Shrivastaw and Singh [43], detection limit for DT–TM complex is 0.2 μg TM. Our results provided detection limit of 0.3 μg TM, and defined calibration dependences were found in the range from 30 to 150 μg mL^{-1} for both peak high and peak surface *(Fig. 3)*. The level of TM in vaccines usually remains in the concentration range from 50 to 100 μg mL^{-1}. Then, the obtained relation covers the range of TM of concentration met in commercial products. Consecutively, for Al, calibration plot in the range of 500–1000 μg mL^{-1} is shown in *Fig. 4*. Determined calibration ranges fully correspond to concentrations present in vaccines.

The full statistical evaluation of the calibration curves, including Mandel's test and lack of fit test results for curvilinearity presence, is given in *Table I*.

Table I. Statistical analysis results for TM and AL calibration curves. Critical values are shown in brackets

Data	Coefficient of determination		$F_{\text{Fisher–Snedecor test}}$		IUPAC *F*-test	Mandel *F*-test	Lack of fit F_{LF} test
	Linear	Quadratic	Linear	Quadratic			
TM	0.99398	0.99887	825.56 (6.61) $F_{calc}>>F_{tab}$	17.31 (6.94) $F_{calc}>F_{tab}$	3.26 (7.71)	17.31 (7.71)	8.731 (3.97)
Al	0.99941	0.99942	15342.00 (5.12)	0.02 (4.46)	−0.11 (5.32)	0.02 (5.32)	0.3867 (2.9)

Conclusions from statistical analysis are as follows

Classical Fisher–Snedecor test [60]. Both regression dependences are linear; however, quadratic influence is constitutive in TM calibration curve $H_p = f(c)$. Nevertheless, calibration curve for Al, $H_p = f(c)$, is purely linear *(Fig. 4)*.

IUPAC *F*-test [61]. There is no reason to reject linearity hypothesis for both of calibration curves. Therefore, regression dependences are assumed linear.

MANDEL *F*-test [61]. TM calibration curve $H_p = f(c)$ is quadratic, and Al calibration curve $H_p = f(c)$ is purely linear.

Lack of fit test for curvilinearity presence [62, 64]: All Al calibration data points are well fitted by linear model of regression curve. Contrary to that, linear model does not fit well TM calibration data points.

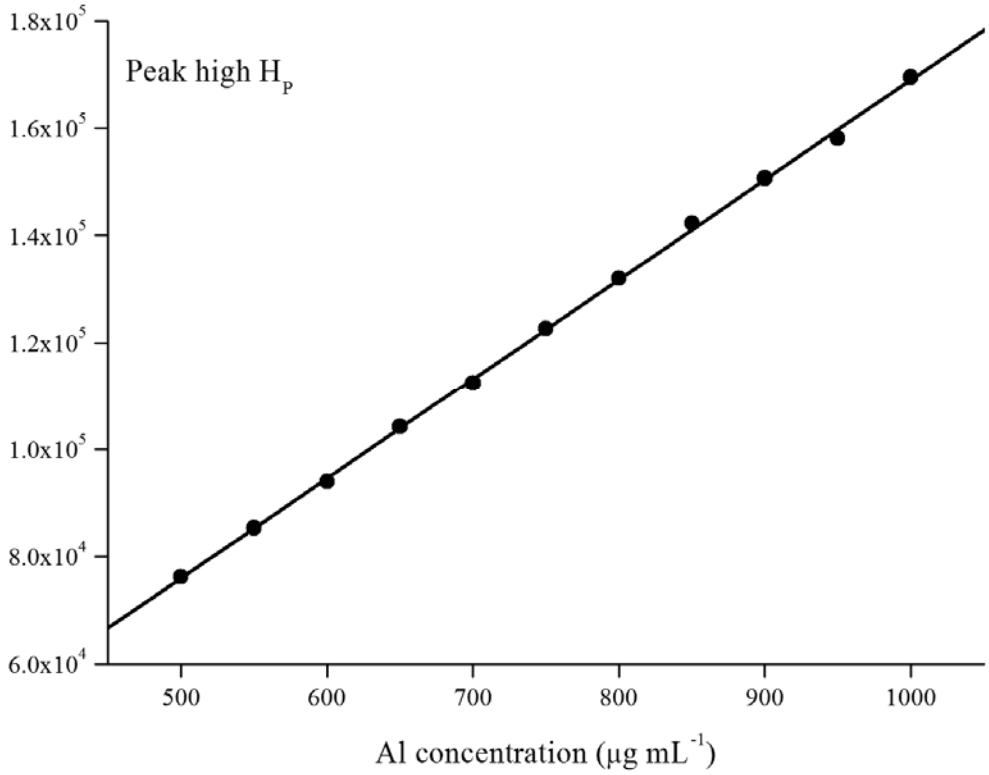

Fig. 4. Calibration plot: peak high Hp vs. Al concentration. Linear regression equations: $H_P = (-1.70114 \cdot 10^4) + (0.0186 \cdot 10^4) \cdot C_{Al}$; $sd_A = 0.1153 \cdot 10^4$; $sd_B = 1.504$; $r = 0.9997$. The results obtained from peak area are almost identical

As a result of the presented statistical analysis, a quadratic model for TM calibration curve *(Table I)* and linear model for Al calibration curve were applied. For TM determination, difference between linear and quadratic model remains within determined confidence interval limit *(Table II)*.

Determined concentrations of TM and Al in investigated original sample of DTP vaccine *(Fig. 5)* are as in *Table II*. Confidence intervals were obtained on the basis of five repeated experiments.

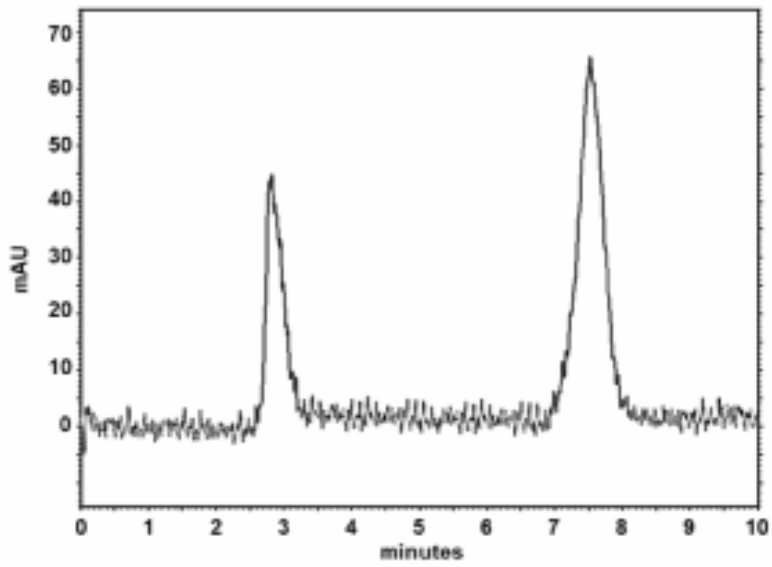

Fig. 5. Chromatogram of DTP vaccine. Retention times: R_t = 7.52 min for TM and R_t = 2.91 min for Al. mAU — milliabsorbance units

Table II. Determined and declared concentrations of TM and Al in DTP vaccine. Confidence intervals were determined on the basis of five independent determinations

	Concentration determined from peak high (averaged) (μg mL^{-1})	Concentration declared (μg mL^{-1})
TM linear model	49.2 ± 0.3	50
TMquadratic model	49.3 ± 0.3	
Al	690.6 ± 0.2	No more than 700

Conclusions

1. RP-HPLC method with the application of postcolumn derivatization with the use of dithizone as complexing agent makes it possible to determine Al and TM simultaneously. The respective peaks of TM and Al are well defined and resolved. The limits of detection for TM and Al are 0.3 and 3 μg,

respectively. Calibration curves cover 30–150 μg mL^{-1} range for TM and 500–1000 μg mL^{-1} for Al. The results obtained from peak area and peak high are almost identical.

2. The presented method can be applied to vaccines, pharmaceuticals, and other products containing TM and Al.

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