

METHOD FOR MEASURING CONTENT OF DTPA IN ADC BY MEANS OF USING LC-MS/MS

FIELD

[0001] The present disclosure relates to the technical field of biomedicine, and in particular to a method for detecting DTPA by LC-MS/MS with high accuracy, strong durability, and good repeatability.

BACKGROUND

[0002] Diethyltriaminepentaacetic acid (DTPA) is an aminopolycarboxylic acid consisting of a diethylenetriamine backbone with five carboxymethyl groups.

[0003] DTPA was widely used in the late 1950s to promote the excretion of radionuclides, and the calcium salts thereof are mainly used at present. The toxic and side effects of DTPA and the related calcium salts thereof mainly include high-dose lethal reactions, liver and kidney injury, intestinal mucosal injury and hematopoietic inhibition (Zhao Xingcheng et al., Toxicity and excretion-promoting application of DTPA, Foreign Medicine (Radiology), 1980-04).

[0004] DTPA is currently widely used in the production of ADC products, and is added in the process of monoclonal antibody reduction, and the main purpose is to protect the reduced protein. DTPA can also be used as a linker for antibody drug conjugates (Virginia del Solar et al., Metal-based antibody drug conjugates. Potential and challenges in their application as targeted therapies in cancer, Journal of Inorganic Biochemistry 199, 2019), and used in the preparation of Platinum metal-based antibody drug conjugates. The process practice shows that without the addition of DTPA, ADC cannot be conjugated normally or the quality of the product after conjugation decreases. There is usually no specific removal process for DTPA in the current ADC production process, and it is only removed during the product dialysis process. Therefore, it has the possibility of residual in the ADC products, which has a certain negative impact on the safety of subsequent drug products, and cannot be ignored. At present, no research on DTPA detection in ADC-related products has been found in the prior art.

[0005] At present, the DTPA detection methods reported in the literature are mainly concentrated on the food field and textile field. Gas chromatography and high performance liquid chromatography are mainly used, and ion chromatography is also used. However, when detected by gas chromatography, DTPA and its complexes are difficult to volatilize due to the presence of polar

carbonyl groups, so they need to be esterified to generate volatile compounds, and the operation is complicated. However, when the HPLC method is used, the detection limit is too high, which is not suitable for the detection of residues. In view of the above reasons, the LC-MSMS method has attracted more and more attention. For example, patent application (CN107315056A) discloses a method for the determination of DTPA-Ca in rat plasma biological samples by LC-MSMS, which achieves excellent chromatographic separation effect and excellent target substance recovery rate. Patent application (CN107167541A) discloses a method for the determination of DTPA-Zn in rat plasma biological samples by LC-MSMS, which also achieves a good separation effect. However, these methods are not suitable for DTPA in antibody-drug conjugate products, because the antibody-drug conjugates, as a mixture of protein biomacromolecules, have a complex system, which seriously interferes with the detection of DTPA.

[0006] LC-MS/MS is actually a comprehensive detection technology that combines LC technology with MS-MS technology. LC-MS/MS is a combination of liquid chromatography (LC) and mass spectrometry (MS). Its working principle is: after the sample is injected, it is first carried by the mobile phase into the chromatographic column, and after separation by the chromatographic column, it enters the mass spectrometer for detection. The mass spectrometer performs detection according to the mass-to-charge ratio (m/z) of the analytes. The analytes are converted into gas-phase ions in the ion source and enter the mass spectrometer. In the triple quadrupole, the primary mass spectrometer scans a specific range of ions or allows specific ions to enter the collision cell. In the collision cell, the molecular ions are fragmented by collision, and the daughter ions are formed and enter into the secondary mass spectrometer. The secondary mass spectrometer scans a specific range of ions or allows specific ions to enter the detector. The advantages of LC-MS/MS are very significant, because gas chromatography can only separate volatile and non-decomposable substances, while liquid chromatography greatly broadens the separation range. It can be seen that the combination of LC and MS/MS with high selectivity and high sensitivity can perform real-time analysis of complex samples. Even in the case of difficult separation by LC, as long as the target compounds are scanned with neutral fragments by MS1 and MS2, the target compounds in the mixture can be found and highlighted, which can significantly improve the signal-to-noise ratio.

[0007] In the method for detecting DTPA by using LC-MSMS, Fe^{3+} derivatization is mostly used. However, the derivatization conditions thereof are all acidic conditions, and mobile phase needs the addition of ion-pair reagents to realize good separation, and the repeatability of the method is poor and unstable. Therefore, in order to detect DTPA better, quickly and efficiently, we developed a method for detecting DTPA by LC-MS/MS based on weak alkaline derivatization

conditions, which effectively solved the difficulties encountered in the detection of DTPA in the prior art. In this application, LC-MSMS detection is used in the detection of DTPA in antibody-drug conjugate products, which has high accuracy, strong durability and good repeatability, and has important application value in the quality control of ADC production.

5

SUMMARY

[0008] The present disclosure uses LC-MS/MS method to detect DTPA in the antibody-drug conjugate product, and FeCl_3 is used for the derivatization of samples under a weak alkaline derivatization condition. The derivatization time is short, and the samples are stable within 8h after
10 derivatization, and a weak basic mobile phase is used simultaneously. Relatively good chromatographic peaks can be produced in a C18 chromatographic column without need for addition of ion-pair reagents. This method is efficient, stable and durable.

[0009] In the first aspect of the present disclosure, it relates to a method for detecting DTPA content in ADC samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS, or
15 LC-MS/MS), wherein the ADC samples detected are derivatized with Fe^{3+} under a weak basic derivatization condition, and a C18 chromatographic column is used while a weak alkaline mobile phase is used, without need for addition of ion-pair reagents.

[0010] Further, the weak alkalinity of the present disclosure refers to $7 \leq \text{pH} \leq 8$.

[0011] Further, FeCl_3 is used for the derivatization with Fe^{3+} .

20 **[0012]** Further, the mobile phase A is an aqueous solution comprising ammonium acetate and ammonium hydroxide; preferably an aqueous solution comprising 0.2% 80-100 mM ammonium acetate (W/V) and 0.01% ammonium hydroxide (W/V) ; most preferably an aqueous solution comprising 0.2% 100 mM ammonium acetate (W/V) and 0.01% ammonium hydroxide (W/V).

25 **[0013]** Further, the mobile phase B is an acetonitrile solution comprising ammonium acetate and ammonium hydroxide; preferably an acetonitrile solution comprising 0.2% 80-100 mM ammonium acetate (W/V) and 0.01% ammonium hydroxide (W/V); most preferably an acetonitrile solution comprising 0.2% 100 mM ammonium acetate (W/V) and 0.01% ammonium hydroxide (W/V).

30 **[0014]** Further, the concentration of the added FeCl_3 solution is about 40-60 $\mu\text{g/ml}$, more preferably 50.0 $\mu\text{g/ml}$.

[0015] The method according to any embodiment of the first aspect of the present disclosure, wherein the method uses DTPA to prepare standard solution.

[0016] Further, the standard solution is preferably a 1.0 µg/ml DTPA solution.

[0017] Further, the preparation of the DTPA solution and the preparation of the series solutions for standard curve comprise the following steps:

[0018] 1) Preparation of DTPA working solution: weighing 5~10 mg of DTPA standard into an EP tube, and dissolving with 1 ml of 0.1% ammonium hydroxide to obtain a mother solution, and then diluting the mother solution stepwise to a DTPA concentration of 1.0 µg/ml;

[0019] 2) preparation of standard curve series solutions: 1.0 µg/ml DTPA standard solution is diluted with mobile phase A to DTPA standard solutions with a series of concentrations of 500 ng/ml, 400 ng/ml, 200 ng/ml, 100 ng/ml, and 50 ng/ml.

[0020] The method further comprises using quality control solutions, which are prepared by diluting 1.0 µg/ml DTPA solution with mobile phase A, and the concentrations of the quality control solutions are 400 ng/ml (HQC), 200 ng/ml (MQC), and 100 ng/ml (LQC) of DTPA quality control solutions.

[0021] According to the method described in any embodiment of the first aspect of the present disclosure, the liquid chromatograph includes but is not limited to: Agilent 1200 liquid chromatograph from Agilent USA, Teledyne Isco high pressure preparative liquid chromatograph, and Agilent HPLC 1260 high performance liquid chromatograph.

[0022] According to the method described in any embodiment of the first aspect of the present disclosure, the tandem mass spectrometer includes but is not limited to: API 4000 tandem mass spectrometer from AB Sciex USA, Shimadzu triple quadrupole liquid chromatography mass spectrometer, and GBC inductively coupled plasma orthogonal acceleration time-of-flight mass spectrometer Optimass.

[0023] According to the method described in any embodiment of the first aspect of the present disclosure, a C18 chromatographic column is used in the liquid chromatography, including but not limited to: YMC-C18, Agilent Extard-C18, and Shiseido PAK CR-18.

[0024] In any embodiment of any aspect of the present disclosure, any technical feature can be applied to the technical feature in other embodiments, as long as they do not contradict. All documents cited in the present disclosure are incorporated herein by reference in entirety, and if the meanings expressed by these documents are inconsistent with the present disclosure, the expression of the present disclosure shall prevail.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] Figure 1: Linear fitting DTPA standard curve

[0026] Figure 2: Determination of DTPA content by LC-MS/MS method

5

DETAILED DESCRIPTION OF EMBODIMENTS

[0027] Example 1 Determination of DTPA content by liquid chromatography-tandem mass spectrometry (LC-MS/MS) method

[0028] 1) Preparation of working solution:

10 **[0029]** Preparation of DTPA working solution: 5-10 mg of DTPA standard was weighed into an EP tube, and dissolved with 1 ml of 0.1% ammonium hydroxide to obtain a mother solution, and then the mother solution was diluted stepwise to a DTPA concentration of 1.0 µg/ml.

[0030] Preparation of ferric chloride working solution: 10~20 mg of ferric chloride was weighed into an EP tube, and dissolved with an appropriate amount of water to obtain a mother solution, and then the mother solution was diluted stepwise to a ferric chloride concentration of 50.0 µg/ml.

15 **[0031]** Preparation of series solutions for standard curve: 1.0 µg/ml DTPA (recorded as A1) standard solution was diluted with mobile phase A according to Table 2 to the DTPA standard solutions with a series of concentrations of 600 ng/ml, 400 ng/ml, 200 ng/ml, 100 ng/ml, and 50 ng/ml. 200 µl of the DTPA standard solutions with different concentrations above were taken, and each was added with 5 µl of ferric chloride solution (concentration of 50.0 µg/ml). Then it was directly tested on the machine after mixed by vortex and reacted at room temperature for 9 h in the dark.

[0032] Table 1

sample name	working solution name	working solution volume (µl)	diluent volume (µl)	total volume (µl)	final DTPA concentration (ng/ml)
STD5	A1	600	400	1000	600
STD4	A1	400	600	1000	400

STD3	STD4	500	500	1000	200
STD2	STD3	500	500	1000	100
STD1	STD2	500	500	1000	50

[0033] Preparation of quality control solutions: 1.0 µg/ml DTPA (recorded as A1', which needs to be freshly diluted from mother solution) standard solution was diluted with mobile phase A according to Table 2 to DTPA quality control solutions with concentrations of 400 ng/ml (HQC), 200 ng/ml (MQC), and 100 ng/ml (LQC). 200 µl of the DTPA quality control solutions with different concentrations above was taken, and each was added with 5 µl of ferric chloride solution (concentration 50.0 µg/ml). Then it was directly tested on the machine after mixed by vortex and reacted at room temperature for 4 h in the dark.

[0034] Table 2

sample name	working solution name	working solution volume (µl)	diluent volume (µl)	total volume (µl)	final DTPA concentration (ng/ml)
HQC	A1'	400	600	1000	400
MQC	HQC	500	500	1000	200
LQC	MQC	500	500	1000	100

[0035] 2) Determination by liquid chromatography-tandem mass spectrometry:

[0036] Treatment of the samples to be tested: Mobile phase A (an aqueous solution comprising 0.2% 100 mM ammonium acetate and 0.01% ammonium hydroxide) was used as a diluent to dilute the ADC samples concentration to an appropriate concentration (the DTPA content of samples should be within the linear range, if it exceeds the range then the dilution fold needs to be increased/decreased for the test again). 200 µl of the diluted samples was taken and added with 5 µl of ferric chloride solution (concentration 50.0 µg/ml), and then directly tested on the machine after mixed by vortex and reacted at room temperature for 4 h in the dark.

[0037] Preparation of mobile phase A: 0.771 g of ammonium acetate was weighed and dissolved by the addition of 80 ml water with stirring, and water was added to make up to 100 ml, and 100 mM ammonium acetate was obtained after the filtration through a GHP filter membrane.

1000 µl of 100 mM ammonium acetate and 500 µl of 10% ammonium hydroxide were measured

precisely, and water was added to the scale of 500 ml, and the mobile phase A was obtained after the filtration through a GHP filter membrane.

[0038] Preparation of mobile phase B: 1000 µl of 100 mM ammonium acetate and 500 µl of 10% ammonium hydroxide were measured precisely, and 500 ml of acetonitrile was added, and the mobile phase B was obtained after the filtration through a GHP filter membrane.

[0039] During detection, mobile phase A and mobile phase B were used in elution according to the concentration gradient shown in Table 3.

[0040] Table 3

elution gradient	elution time (min)	module	event	parameter
	0.0	Pumps	Pump B ConC	10
	2.0	Pumps	Pump B ConC	10
	2.5	Pumps	Pump B ConC	50
	5.0	Pumps	Pump B ConC	80
	5.5	Pumps	Pump B ConC	10
	8.0	Controller	Stop	/

[0041] 3) Data processing and analysis

[0042] Evaluation standard: Standard curve: $r \geq 0.9800$, the recovery rate of the concentration points of the upper limit of quantification and lower limit of quantification is within 80.0%-120.0%.

[0043] The recovery rate of the quality control is within 80.0%-120.0%. At least 50% of the quality control samples of each concentration should meet this range, and at least 4 quality control concentration points in the same analysis batch should meet the requirements.

[0044] The specific results were as follows:

[0045] Table 4: Standard curve (see Figure 1 for the specific fitting curve)

sample name	sample type	actual concentration (ng/ml)	peak area	retention time	calculated concentration (ng/ml)	accuracy
STD1	Standard	50	2.16E+04	2.57	45.481	90.96
STD2	Standard	100	1.32E+06	2.56	118.126	118.13
STD3	Standard	200	2.94E+06	2.57	209.537	104.77
STD4	Standard	400	6.36E+06	2.58	401.707	100.43
STD5	Standard	500	7.69E+06	2.58	476.081	95.22
STD6	Standard	600	8.88E+06	2.58	543.008	90.5
LQC	QC	100	1.200e6	2.58	111.701	111.70
MQC	QC	200	2.865e6	2.58	205.222	102.61
HQC	QC	400	5.924e6	2.59	377.034	94.26

[0046] The standard curve obtained after linear fitting is as follows:

[0047] $Y=16058x-416744$ $R^2=0.9936$ According to the results shown in Figure 1, $R^2>0.99$, the fitting were relatively good.

[0048] Example 2. Detection method results and evaluation

5 **[0049]** 1) Durability (stability)

[0050] Durability of derivatization temperature: 200 μ l of DTPA standard solution with a concentration of 600 ng/ml was taken and added with 5 μ l of ferric chloride solution (concentration 50.0 μ g/ml), and mixed by vortex and reacted in the dark for 4 hours. The derivatization temperatures were room temperature, 37°C and 50°C, respectively, and the influence of the
10 derivatization temperature on the results was investigated. The specific results are shown in Table 5.

[0051] Table 5

theoretical concentration of sample (ng/ml)	concentration of sample derivatized at 37°C (ng/ml)	concentration of sample derivatized at room temperature (ng/ml)	concentration of sample derivatized at 50°C (ng/ml)	RSD (%)
50	59.827	46.434	71.934	21.5
100	104.847	117.73	107.631	6.2
200	213.285	192.906	190.428	6.3
400	375.96	386.096	449.338	9.9
500	444.249	465.884	411.396	6.3
600	579.645	619.491	657.508	6.3

[0052] Durability of derivatization time: 200 μ l of DTPA standard solution with a concentration of 600 ng/ml was taken and added with 5 μ l of ferric chloride solution (concentration 50.0 μ g/ml), and mixed by vortex and reacted in the dark at room temperature. 4 hours, 6 hours, and
15 8 hours were used respectively to investigate the influence of the derivatization time on the results. The specific results are shown in Table 6.

[0053] Table 6

theoretical concentration of sample (ng/ml)	concentration of sample derivatized for 4h (ng/ml)	concentration of sample derivatized for 6h (ng/ml)	concentration of sample derivatized for 8h (ng/ml)	RSD (%)
50	46.434	51.557	45.159	7.1
100	117.73	101.666	93.884	11.6
200	192.906	139.134	132.229	21.5
400	386.096	329.076	290.415	14.4
500	465.884	458.652	489.751	3.5
600	619.491	666.944	700.338	6.15

[0054] The stability of the tested solutions (samples for precision test), *i.e.*, the content change of standard DTPA at 0 hours, 4 hours, and 8 hours after the derivatization reaction of DTPA and ferric chloride was investigated. The specific results are shown in Table 7.

[0055] Table 7

theoretical concentration of sample (ng/ml)	concentration of sample at 0h (ng/ml)	concentration of sample at 4h (ng/ml)	concentration of sample at 8h (ng/ml)	RSD (%)
50	52.775	56.24	58.383	5.1
200	195.657	170.057	180.946	7.1
400	342.436	338.507	337.147	0.8
600	734.414	640.083	642.08	8.0

- 5 **[0056]** Evaluation standard: Concentration (relative standard deviation RSD%) $\leq 25.0\%$. According to the experimental results in Tables 5-7, the RSD% is less than 25.0%, so the method has strong durability.

[0057] 2) Repeatability

- 10 **[0058]** After the instrument was stabilized, the standard 3 solution (DTPA concentration of 200 ng/ml) was taken and derivatized according to the experimental method, and injected for analysis. The results of 6 samples tested were evaluated, and the results were shown in Table 8. The chromatograms were collected, and the RSD of the main peak content was calculated.

[0059] Table 8

number of injections	peak area (EU)	concentration (ng/ml)
1	1.37E+05	213.430
2	1.15E+05	177.924
3	1.40E+05	218.562
4	1.42E+05	220.844
5	1.45E+05	225.477
6	1.45E+05	226.486
RSD	8.3%	8.5%

- 15 **[0060]** In the field of quantitative analysis, repeatability refers to the precision of the results obtained by the same analyst within a short interval under the same operating conditions. The acceptable standard for repeatability is: the main peak concentration RSD $\leq 15.0\%$, this test RSD

<15.0%, and the RSD of this method is much smaller than the highest ratio, and the repeatability is good.

[0061] The spirit of the present disclosure has been described in detail above through the preferred examples of the present disclosure. Those skilled in the art understand that any changes, equivalent changes and modifications made to the above embodiments according to the technical essence of the present disclosure all fall within the protection scope of the present disclosure.

10

15

20

25

CLAIMS

1. A method for detecting DTPA content in ADC by liquid chromatography-tandem mass spectrometry (LC-MS/MS), wherein ADC samples detected are derivatized with Fe^{3+} under a weak basic derivatization condition, and a C18 chromatographic column is used while a weak alkaline mobile phase is used, without need for addition of ion-pair reagents.

2. The method according to claim 1, wherein FeCl_3 is used for the derivatization with Fe^{3+} .

3. The method according to claim 2, wherein the weak basic mobile phase comprises mobile phase A, and the mobile phase A is an aqueous solution comprising ammonium acetate and ammonium hydroxide; preferably an aqueous solution comprising 0.2% 80-100 mM ammonium acetate and 0.01% ammonium hydroxide; most preferably an aqueous solution comprising 0.2% 100 mM ammonium acetate and 0.01% ammonium hydroxide.

4. The method according to claim 3, wherein the weak basic mobile phase comprises mobile phase B, and the mobile phase B is an acetonitrile solution comprising ammonium acetate and ammonium hydroxide; preferably an acetonitrile solution comprising 0.2% 80-100 mM ammonium acetate and 0.01% ammonium hydroxide; most preferably an acetonitrile solution comprising 0.2% 100 mM ammonium acetate and 0.01% ammonium hydroxide.

5. The method according to claim 4, wherein the added FeCl_3 solution has a concentration of about 40-60 $\mu\text{g/ml}$, more preferably 50.0 $\mu\text{g/ml}$.

6. The method according to claim 5, wherein the method uses DTPA to prepare standard solution.

7. The method according to claim 6, wherein the standard solution is preferably a DTPA solution of 1.0 $\mu\text{g/ml}$.

8. The method according to claim 7, wherein the preparation of the DTPA solution and the preparation of the series solutions for standard curve comprise the following steps:

1) Preparation of DTPA working solution: weighing 5~10 mg of DTPA standard into an EP tube, and dissolving with 1 ml of 0.1% ammonium hydroxide to obtain a mother solution, and then diluting the mother solution stepwise to a DTPA concentration of 1.0 $\mu\text{g/ml}$;

2) Preparation of standard curve series solutions: 1.0 $\mu\text{g/ml}$ DTPA standard solution is diluted with mobile phase A to DTPA standard solutions with a series of concentrations of 500 ng/ml, 400 ng/ml, 200 ng/ml, 100 ng/ml, and 50 ng/ml.

9. The method according to claim 6, wherein the method further comprises using quality control solutions, which are prepared by diluting 1.0 µg/ml DTPA solution with mobile phase A, and the concentrations of the quality control solutions are 400 ng/ml (HQC), 200 ng/ml ml (MQC), and 100 ng/ml (LQC) of DTPA quality control solutions.

5 10. The method according to claim 1, wherein the liquid chromatograph includes but is not limited to: Agilent 1200 liquid chromatograph from Agilent USA, Teledyne Isco high pressure preparative liquid chromatograph, and Agilent HPLC 1260 high performance liquid chromatograph.

10 11. The method according to claim 1, wherein the tandem mass spectrometer includes but is not limited to: API 4000 tandem mass spectrometer from AB Sciex USA, Shimadzu triple quadrupole liquid chromatography mass spectrometer, and GBC inductively coupled plasma orthogonal acceleration time-of-flight mass spectrometer Optimass.

12. The method according to claim 1, wherein the C18 chromatographic column includes but is not limited to: YMC-C18, Agilent Extard-C18, and Shiseido PAK CR-18.