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Internal Standard an Important Analyte Use in Drug Analysis by Liquid Chromatography Mass Spectrometry- An Article

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ABSTRACT

Internal standard is an external compound which is mixed with targeted analytical solution and matrix as a constant concentration and use for preparing calibration standard curve by using ratio of analyte area and internal standard area with analyte concentration and internal standard concentration. This calibration curve used for quantification of unknown concentration of anlayte of interest. This article provide necessary information about internal standard like its selection procedure, characterization, types and response factor , to all analyst who are connected with drug analysis. This article is more important and I think first article which focuses a clear idea about internal standard use in drug analysis.

ARTICLE DETAILS

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ABBREVIATION

LC-MS: Liquid Chromatography Mass Spectrometry; RSD: Relative standard deviation; RF: Response factor; RRF: Relative response factor; RT: Retention time; RRT: Relative retention time; RTS: Retention time shifts;

1. INTRODUCTION

A known amount of compound which is added to an unknown sample known as an internal standard. In analytical and bio-analytical chemistry, calibration curve (standard curve) used in a method for determining the concentration of an unknown sample by comparing a set of standard known concentrated sample. Calibration curve use for instrumental calibration but another approach for uses of calibration curve for standardization by mixing the standard into the unknown sample, which giving an internal standard. This substance added in a constant amount to a blank and standard sample in an analysis and compensate for both systematic errors like matrix effect of the solvent, effect is equal forBoth of standard and the analyte, the ratio is unaffected and random errors like fluctuation of the instrument are expected that the ratio of the signals of the standard and analyte does not change. [01] The limitation of internal standards is hard to search a suitable internal standard, because the internal standard must have a signal that is similar to the analyte but if different enough it can distinguish by theinstrument. The concentration of the internal standard is constant in the sample and this concentration ofthe internal standard should not suppress or enhance the signal of the analyte. [02]

Chromatography is the separation of the chemical components or analyte in a mixture which contain oneliquid phase called mobile phase and other is solid phase called stationary phase. Among various others chromatography techniques one of the technique is liquid chromatography mass spectrometry. Here alsorequire individual standards and reproducible conditions enable peak identification by retention time and internal standard also require for quantitation purpose. Internal standard is important for quantitation in High performance liquid chromatography and gas chromatography, liquid mass spectrometry. So, internal standard is a very important analyte for quantitation of

individual component by chromatographically, but choice of internal standard is very important and difficult work for this purpose because of interaction between the molecules. In this article discuss about the main characteristics of internal standard by which we select the suitable internal standard according to characteristics of targeted analyte and also discuss about the name and its characteristics of some general internal standard and some calculation which help me for selecting the suitable internal standard. [03]

2. LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

This article applies to the quantitation of small molecules using mass spectrometry in pharmacokinetic analysis and same principles can also be applied to the quantitation of peptides and proteins in biologicalmatrices. Before the advent of LC-MS quantitation was accomplished using HPLC and UV detection. Pharmacokinetic analysis on HPLC depends on retention time, peak area and UV spectral character but this assay suffered from lack of sensitivity and specificity which time to time mislead the investigator. Mass Spectrometry characterization is a new tool in pharmacokinetic analysis. Mass Spectrometry has ability to separate the analytical molecules according to related molecular mass and permits their detection and quantitation with extreme sensitivity and performance liquid chromatography which facilitates the rapid and quantitative separation of compound from each other and from the other constituents of complex mixtures and matrices by their physic-chemical properties because although mass spectrometry is a very sensitive method of analysis and highly selective but it is necessary to isolate the target analyte from thousands of different molecules. Mass spectrometry has ability todifferentiate the compounds because it can differentiate the compounds by their mass-to- charge ratio which is insufficient to most of the practical application.

The best way of performing quantitation is by using a mass spectrometer capable of MS/MS fragmentation and is commonly accomplished with a triple quadruple or ion trap mass spectrometer. This fragmentation is required because many compounds have same intact of mass and the technique of quantitaion by using first dimension of MS suffers from lack of specificity in a complex matrix like blood and MS2 fragmentation provides a unique fragment. Liquid Chromatography Triple Quadrupole Tandem Mass Spectrometry methodologies have largely utilized calibration curve-based quantitaion ona batch basis. Internal standard concentration with analyte concentration and internal standard peak area with analyte peak area ratio use for quantitation purpose. [04]

3. INTERNAL STANDARD

An internal standard should be used when performing mass quantitaion and an appropriate internal standard requires for controlling extraction, HPLC injection and ionization variability, so it work by normalizing for differences for extraction, injection, chromatography, ionization and detection between samples. These compounds are utilized across a wide range of mass spectrometry application including therapeutic drug monitoring, newborn screening, endocrinology and pain management testing and especially useful in improving the accuracy of quantitaion in complex matrices. In a complex matrix twodifferent standard levels give identical response especially at the low end of the curve but not identified at this two point but if internal standard is used then this two points can be differentiated. To prepare a standard curve an internal standard should be used because without an internal standard find moderate success because without an internal standard % relative standard deviations(RSDs) of replicates can be as high as 20% but with an internal standard the %RSDs can be brought down to approximately 2%. It involves the comparison of the instrument responses from the target compounds in the sample to the responses of reference standards added to the sample or sample extract before extract. The reference standard normalized the response of the targeted molecule. This reference standard (internal standard) contained within the aliquot of the sample extract that is actually injected into the analytical instrument. [05]

[Figure: 1]

A constant concentration of the internal standard is added to all extracts. Each calibration curve containsratio of peak area of the analyte and internal standard and also use the height of the peak of the analyte and internal standard. The ratio is the response factor (RF) or relative response factor (RRF) which is thetarget compound response is calculated with the internal standard.

[Figure: 2]

Response factor (RF) = [(A$_X$) (C$_{is}$)]/[(A$_{is}$) (C$_X$)]

Where,

A_X = Area of the compound

C_X = Concentration of the compound

A_{is} = Area of internal standard

C_{is} = Concentration of the internal standard

This response factor use for determination of concentration of internal standard and analyte in diluted sample after dilution and also use determination of concentration of internal standard in unknown sample.

Determination of concentration of internal standard (C_{is}) in diluted sample after dilution

Concentration internal standard in stock solution*volume of stock solution/volume of diluted solution

Determination of concentration of internal standard (C_{is}) in unknown sample

[06] Concentration of analyte in diluted sample* volume of diluted solution/ volume of stock solution

Standard is added to the plasma sample by spiking and a known analyte with concentration gradient is added to the plasma sample. After injecting to the mass spectrometry signal of this known analyte is measured which determine the concentration of the original sample because signal is proportional to the concentration of analyte.

If C_i = Initial concentration of analyte before adding standard, C_f = Unknown concentration of analyte after adding standard,C_S = Known concentration of standard after adding standard, S_i = Initial concentration of standard before adding standard,

Then, $\mathbf{C_i / (C_f + C_S) = I_X / I_{X+S}}$ where $\mathbf{C_f = C_i * V_0/V}$ and $\mathbf{C_S = S_i * V_S/V}$ where $\mathbf{V= V_0 + V_S}$

V_0 = Initial volume of unknown sample,V_S = Volume of standard added,

V = Total volume of sample.

Injection of the sample to the chromatography is the largest sources of errorless. During manual injection of small volume of sample 1microlitre by injector then error notified during injection, so there are uncertainties in reproducibility injecting this small volume of sample, often a couple of percent relative standard deviation.

For quantification of analyte in liquid chromatography mass spectrometry using linear regression and equation is **Y = mx + c**, where m is the response factor, x is concentration, c is represent as a baseline response constant and Y is the response. But with internal standard this equation is

RRF + c = response ratio

Where RRF is the relative response factor as a concentration ratio.

But baseline response does not affect the ratio of analyte and internal standard responses, so, **RRF= response ratio**

Therefore, **RRF (analyte concentration/internal standard concentration) = analyte response/ internal standard response**

Analyte concentration = (analyte response * internal standard concentration) / (internal standard response *RRF)

By using multiple data points in a calibration curve RF and c are found in linear regression quantitative method. Thumb rule is that the internal standard should be used to the lower 1/3 of the working standardcurve.

4. TYPES OF INTERNAL STANDARDS: [07]

The internal standards uses in liquid chromatography mass spectrometry are five types- **[Table: 1] Structural Analogs internal standard:** These are similar in structure to an analyte.

i. **Stable label analogs internal standard:** These are typically Deuterium, ^{13}C, and N^{15}

ii. **Structurally unique(exogenous) internal standard:** It is not observed in samples

iii. **No H/D Exchange internal standard**: It checks the stability in solution and ion source.

iv. **Structurally dissimilar internal standard:** It is uses when structurally similar are not available.

5. SELECTION CRITERIA OF INTERNAL STANDARD: [08]

In quantitative LC-MS, internal standard is a carefully chosen known concentrated amount of compoundwhich is different from analyte and spiked in the analytical sample. Internal standard should be mixed asearly as possible in the analytical procedure for correction of effects. But in some application like in methods which have digestion stage, internal standard should be added in later stage because after digestion stage the analogues internal standard adds to the target compound. Some important criteria should be maintained for the selection of internal standard. These are

i. Internal standard should be depends on the mode of ionization and also depends on MS/MS platform.

ii. The ionization response and fragmentation pattern of internal standard compound should be similar tothe analyte.

iii. The differentiation of mass should be adequate in response.

iv. The multiple reaction monitoring transitions of internal standard should not interfere with multiple reaction monitoring transitions of analyte.

v. Internal standard compound should not be present in any of the analytical samples to be analyzed andsimilar in analytical behavior.

vi. According to chromatography internal standard should be identical to analyte which is quantified forstable label analogs internal standard.

vii. In the case isobaric structural analogs internal standard should be resolved chromatographically from analyte of interest.

viii. For non-isotopically labeled internal standard should be same physicochemical properties to the targetanalyte and should be elute as close to the

target analyte as possible.

ix. The ideal compound of internal standard should be isomers, homologues, and structural analogues of thetarget analyte.

x. The quantitaion of the internal standard should not be affected by concentration oftarget analytes,surrogates and by matrix interferences.

xi. Internal standard should be pure otherwise impurities cause interferences with analyte or other analytesin the test sample.

xii. In case of isotopic internal standard it should be isotopically pure and ratio is $M0/Mn$

xiii. Isotopic distribution should be adjusted for natural abundance of isotopes and it should be distributed like $M0$…..Mn, $Mn+1$, $Mn+2$ and which is mostly important for analytes with chlorine, bromine, and sulphur. If multiple internal standard use in the same analysis then carefully check the distribution of isotope because it vary from lot to lot.

xiv. If the prepared bio-analytical methods with wide dynamic range and high sensitivity that is lower quantitation levels then high purity, co-elute with the analyte of internal standard should be required which exhibit minimal or no scrambling or cross talk.

xv. If isotopic internal standard are unavailable or expensive and time consuming then use analytical compound analogue which should be tested and quantified similar compound or slightly different by parent mass.

xvi. For quantification of small molecule a common internal standard is a chlorinated version of the parent molecule which has similar chromatographic retention time.

6. PROCEDURE OF USING INTERNAL STANDARD IN QUANTIFICATION

[09] Procedure of internal standard quantification illustrated with 5 steps.

i. Proper handling of sample by making a sample solution

a. Weigh accurately

ii. Internal standard calibration curve preparation

iii. Preparation of targeted sample with internal standard for LC-MS/MS

iv. Run the prepared sample and calculate the concentration of the sample

v. Results: LC-MS/MS analysis of targeted sample with internal standard

7. HOW DOES INTERNAL STANDARD WORK? [10]

Internal standard is an important analyte or aid for the quantification of an unknown analyte in LC-MS/MS especially when used extraction procedure including various steps and when loss of the sample in volumetric ratio. In the analytical sample add a constant concentration of internal standard in each sample and calculate the area and height of internal standard and unknown analyte then use in ratio of area and height of internal standard and analyte and these ratio are used in the calibration process in preparation of calibration curve **[Figure: 3]**

For the preparation of calibration curve Y axis contain ratio of analyte area with internal standard area and X axis contain ratio of analyte concentration and internal standard concentration. When unknown samples are run then area of the analyte and internal standard measured and ratio of the area use for the calculation of the concentration of the unknown analyte. During liquid liquid extraction, when reconstituted the extraction residue and evaporate to dryness of an organic phase then internal standard helps to correct the sample volume for uncontrollable change. Again if error occurs during injection of the solvent then it will be managed because ratio of analyte and internal standard will be same. Internal standard help in analysis **[Figure: 4]**

i. To correct the volumetric recovery errors in the sample preparation.

ii. To construction a calibration curve by using constant amount of concentration to each calibrator and calibration curve form by using ratio of area of analyte and internal standard with ratio of analyte concentration and internal standard.

iii. We determine the unknown concentration of analyte in each sample by using calibration curve which are formed by ratio of analyte area and internal standard area with ratio of analyte concentration and internal standard.

8. BENEFITS OF USE OF INTERNAL STANDARD: [11]

a. It helps for routine variation in the response of the chromatographic system.

b. It helps to quantify variations in the exact volume of sample extract introduced into the chromatographicsystem.

c. The RT of the analyte and the internal standard may be used to calculate the RRT of the target compoundand helps to compensate for small RTS.

d. It helps in regulation of bio-analysis, which is putting in a place during method development of particular analyte.

e. Injection variance

f. Normalization of recovery differences

g. Normalization of ionization effects between calibrators

and samples.

h. One of the most valuable components in LC-MS/MS analytical quality.

9. DISADVANTAGES OF USE OF INTERNAL STANDARD: [12]

The principal limitations of internal standard calibration are that the internal standard should be compounds that are not found in the samples to be analyzed and they must produce an unambiguous response on the chromatographic detector system.

10. WHAT ARE THE SOURCES OF INTERNAL STANDARD RESPONSE VARIABILITY? [13]

By design, the intention is to add the same concetration of IS to all samples. The variation of internal standard responses among samples is found and among running samples analyzed in the same batch. The internal standard responses variation observed for human errors due to sample processing, instrumental matter that may occur during analysis time, and matrix effects.

11. WHEN IS INTERNAL STANDARD RESPONSE VARIABILITY NOT LIKELY TO IMPACT THE ACCURACY OF THE DATA? [13]

The variable reponse of internal standard does not affect the accuracy of the data when the range of internal standard responses for unknown samples is similar to the range of internal standard responses for calibration concentrations and quality control samples in the same analytical run.

12. WHEN CAN INTERNAL STANDARD RESPONSE VARIABILITY IMPACT THE ACCURACY OF THE DATA? [13]

The responses of variation of internal standard does not affect the accuracy of the data when the range of the internal standard responses for unknown samples is different than the range of internal standard responses for calibration concentrations and quality control samples in the same run.

13. EXAMPLES OF SOME INTERNAL STANDARD WHICH ARE USED IN DRUG ANALYSIS IN LC-MS/MS

Examples of some internal standards which are used according to mode of ionization:

Tolbutamide (Negative mode)

Propranolol (Positive mode)

Atorvastatin (Negative mode)

Letrozole (Negative mode)

Salicylic acid (Negative mode)

Clopidogrel carboxylic acid (Positive mode)

Metoprolol (Positive mode)

Clonazepam (Positive mode)

Itraconazole (Positive mode)

Benidipine (Positive mode)

Glimeperide (Positive mode)

Teneligliptin (Positive mode)

Naringenin (Negative mode)

Telmisartan (Positive mode)

Losartan carboxylic acid (Positive mode)

Azilsartan (Positive mode)

Irbesartan (Negative mode)

14. CONCLUSION

Internal standards are important analyte for analysis in liquid chromatography and also in other instruments.This article will be helpful to provide necessary information about internal standard to all analyst.

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LIST OF FIGURES

1. Figure 1: picture of internal standard

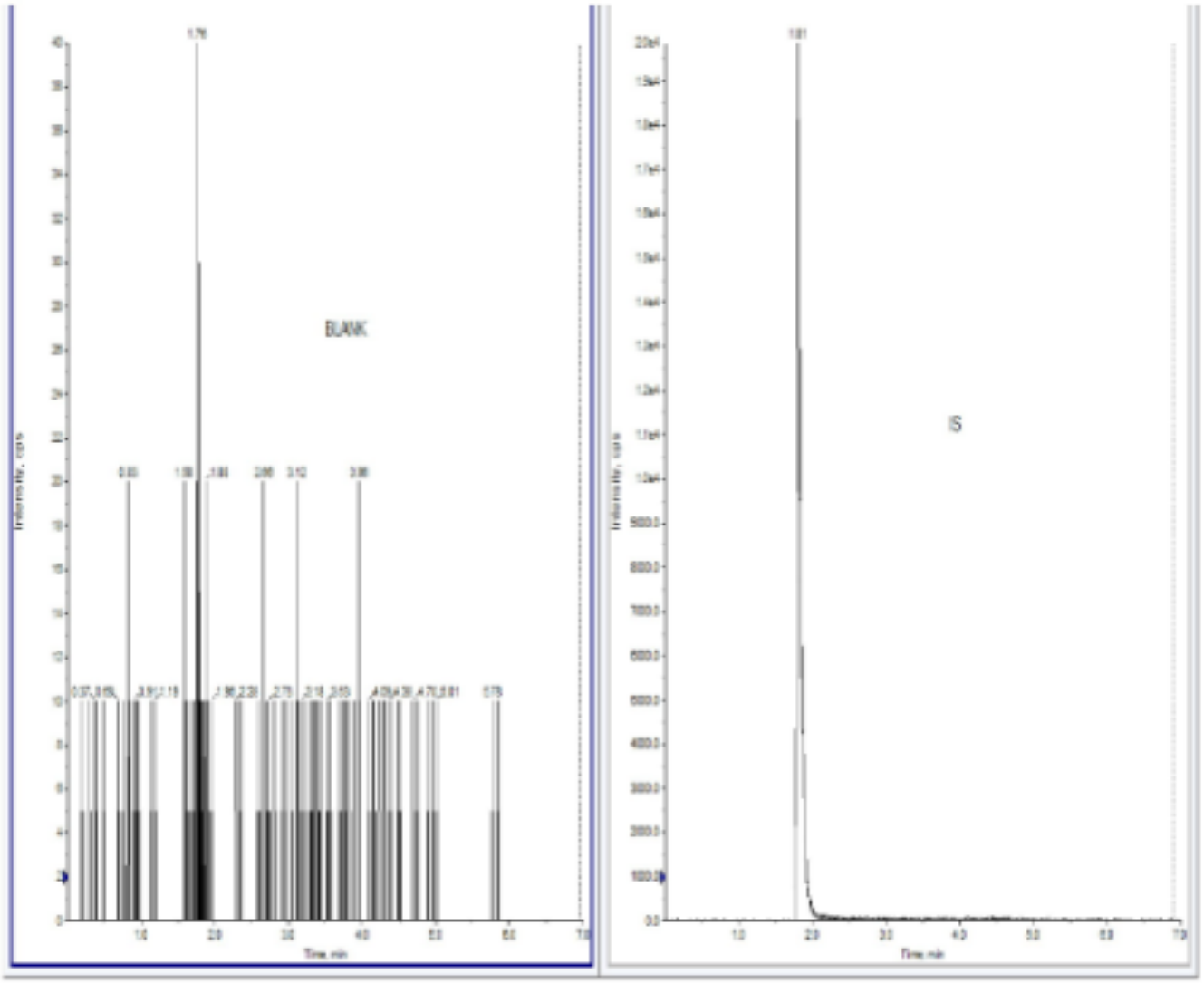

Figure-1: Picture of internal standard

2. Figure 2: Response factor

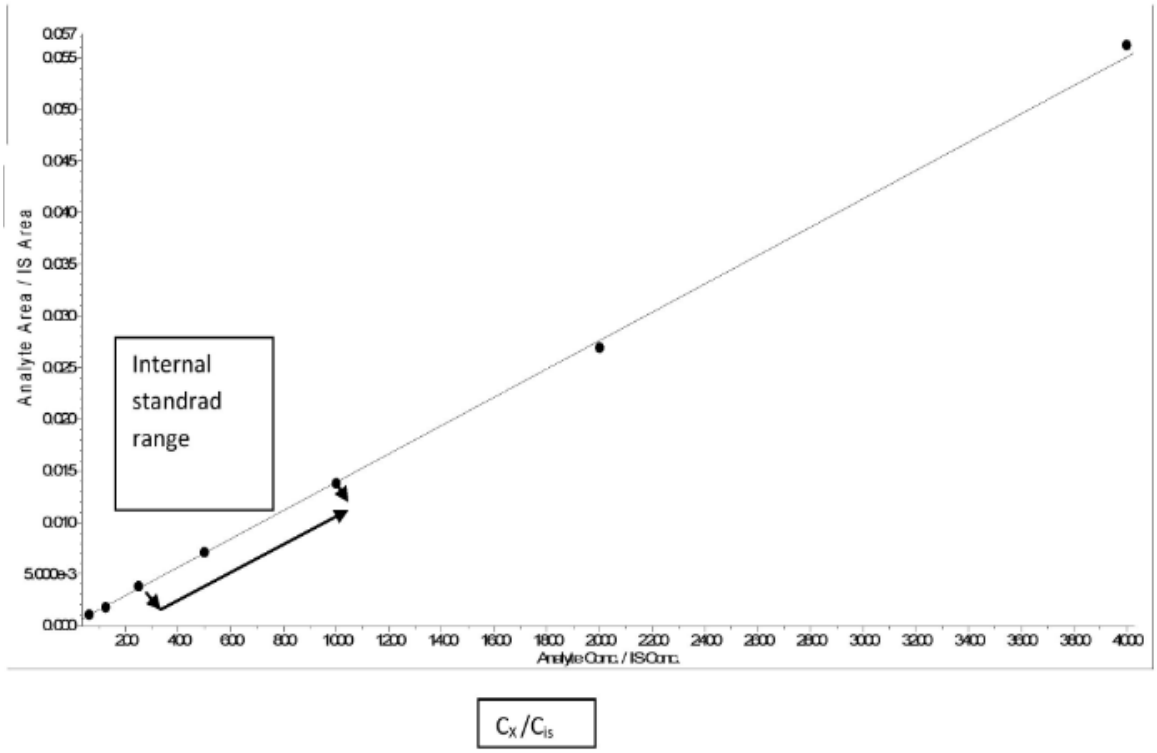

Figure: 2 Response factor by calibration curve

3. Figure 3: Calibration curve by using internal standard

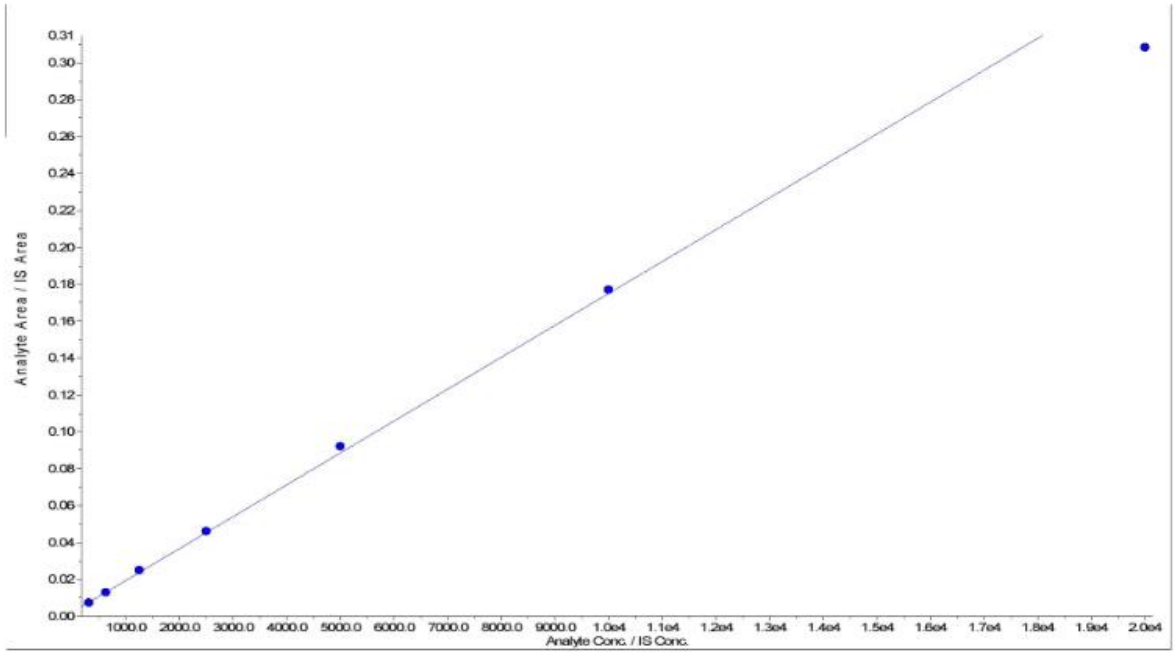

Figure: 3 Calibration curve by using internal standard

4. Figure 4: Internal standard working curve

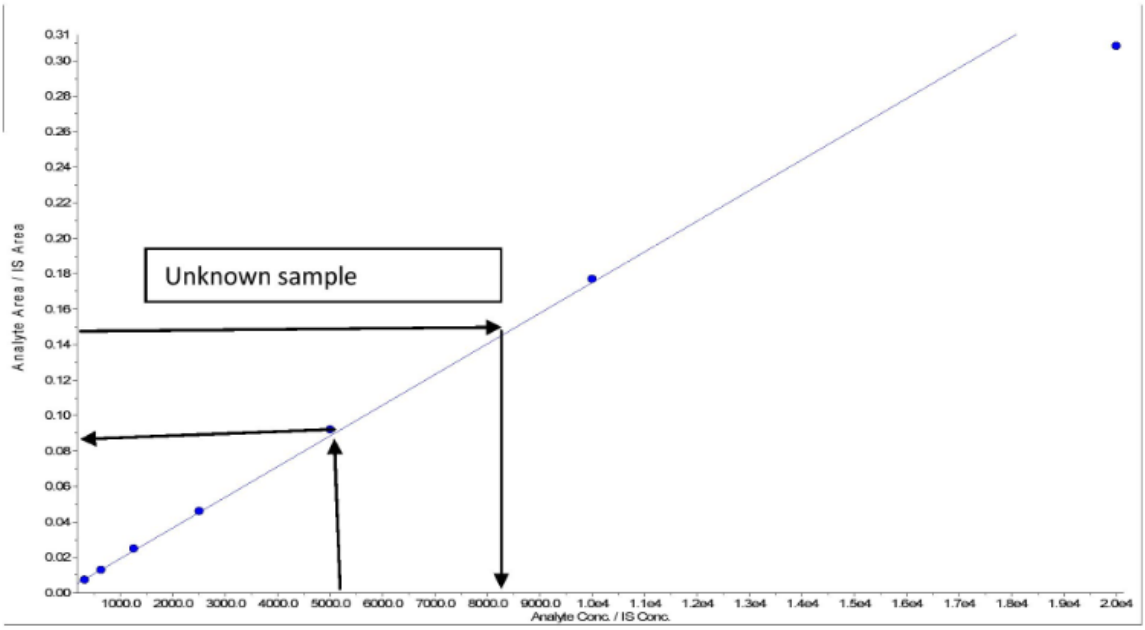

Figure: 4 Internal standard working curve

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List of Table:

1. Table: Types of internal standards

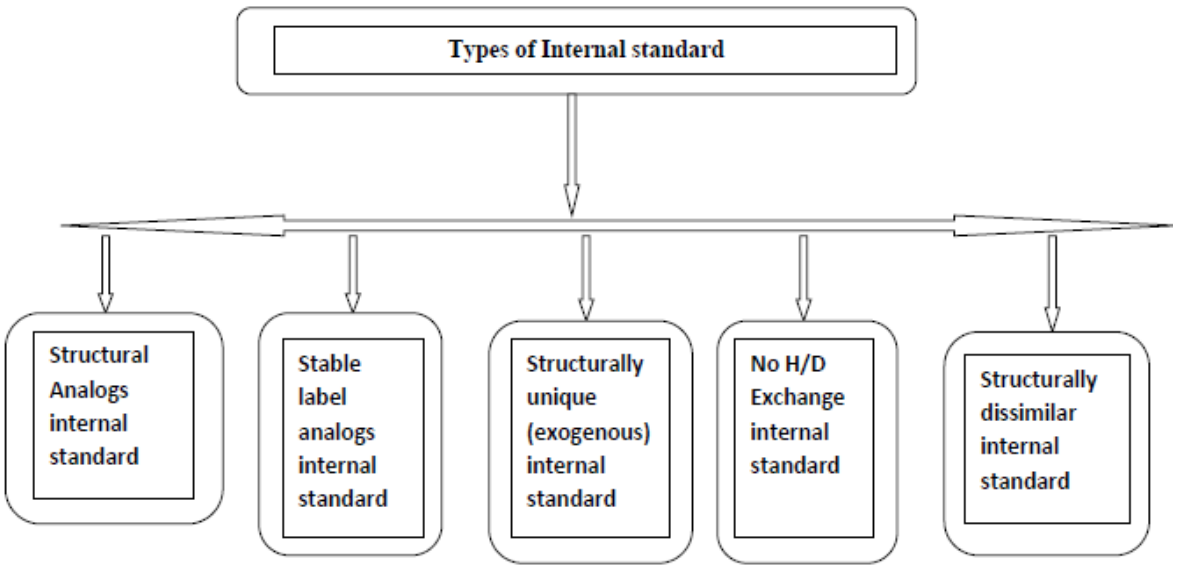

Table: 1 Types of internal standard