Research Article

Development and Validation of a Liquid Chromatography-Tandem Mass Spectrometry Method for Direct Determination of Tetrahydrobiopterin - An Enzyme Cofactor in Human Plasma

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ABSTRACT

Tetrahydrobiopterin (BH4) is a naturally occurring pteridine and cofactor for a variety of enzymes, used for the treatment of Phenylketonuria an autosomal recessive metabolic disorder characterized by hyperphenylalaninemia in association with neurocognitive and neuromotor impairment. BH4 is readily oxidized to dihydrobiopterin (BH2) and biopterin (B), however only BH4 can provide proper cofactor functions. A high performance liquid chromatography-mass spectrometric method employing positive electrospray ionization was developed for the determination of BH4 concentration in human plasma. BH4 together with the internal standard (Metaxalone) was extracted from 0.2 mL of human plasma by protein precipitation using acetonitrile. The chromatography was performed using a Hypurity Cyano column with the mobile phase consisting of Acetonitrile and 5mM Ammonium acetate buffer solution. The method was validated as per USFDA guidelines. Standard curves were linear over the concentration range 1.000–100.000 ng/mL ($r > 0.9990$) and the limit of quantification was 1.000 ng/mL. Within-run and between-run coefficients of variation were <9.52% and mean accuracy ranged from 89.55% to 99.41%. The present method provides a validated, simple, robust, fast and sensitive analytical tool for quantifying BH4 in human plasma and can be applied to a clinical pharmacokinetic study.

Keywords: Tetrahydrobiopterin; LC-MS/MS; method validation; human plasma; pharmacokinetics.

INTRODUCTION

Tetrahydrobiopterin (BH4) is a coenzyme and contributes to the biosynthesis of monoamine neurotransmitters [dopamine, norepinephrine, epinephrine, and serotonin]. 6R-BH4 is the naturally occurring pteridine and is only biochemically active in the enantiomeric R form. BH4 is biosynthesized from guanosine triphosphate (B Thöny et al., 2000). BH4 is playing a significant role in treatment of Phenylketonuria (PKU) (Michals-Matalon et al., 2008) and BH4 deficiencies. PKU is an autosomal recessive metabolic disorder characterized by hyperphenylalaninemia in association with neurocognitive and neuromotor impairment. BH4 deficiency results in monoamine and nitric oxide neurotransmitter deficiency or chemical imbalance. Since nitric oxide production is important in regulation of blood pressure and blood flow, thereby playing a significant role in cardiovascular diseases, tetrahydrobiopterin is a potential therapeutic target (Khoo J et al., 2005).

Sapropterin dihydrochloride is a synthetic formulation of 6-R-L-erythro-5, 6, 7, 8-tetrahydrobiopterin (BH4) being introduced in the US for treatment of PKU. When 6R-BH4 is used in vivo as a cofactor, it is converted to quinoid dihydrobiopterin (R-q-DHBP) and is metabolized to dihydrobiopterin (DHBP) and biopterin (BP). R-q-DHBP and DHBP are reduced by pteridine reductase and folate reductase to 6R-BH4, which is recycled. Plasma protein binding studies in rats and in humans revealed no significant binding of total biopterins to plasma proteins (Novato et al., 2007). BH4 is included in list of Drug Substances and Drug Products seeking monographs USP updated on October 11, 2011. The general view during the initial phase of investigations was that BH4 acts as radical scavenger. BH4 has also participated in clinical trials studying other approaches to solving conditions resultant from a deficiency of tetrahydrobiopterin. These include autism, hypertension, endothelial dysfunction, and chronic kidney disease (Novato et al., 2010).

Till date, no LC-MS/MS method has been published for direct estimation of tetrahydrobiopterin (BH4) in human plasma samples at a sensitivity of 1.000 ng/mL. As BH4 is highly unstable and readily oxidised to Dihydrobiopterin and Biopterin, few indirect LC-MS/MS methods are reported (Helmut et al., 2006 and Zhao Y et al., 2009), in which its oxidation product were measured and then conversion ratio was applied to calculate the BH4 concentrations. These methods need careful assessment of BH4 conversion.

Other assay methods, such as HPLC–diode array, electrochemical, fluorescence and diode array detector were also developed to detect BH4 in bio samples, but are complex due to one or the other reasons like indirect methods involving enzymatic or post column oxidation and complex conversion calculations, direct methods using more than one detector and with longer run times (Kim HL et al., 2010, Fukushima et al., Cañada et al., 2009,S. Sianna et al., 2008 and Tani Y et al., 1993).

To facilitate clinical pharmacokinetic study of BH4, a simple, sensitive, specific, fast and rugged high performance liquid chromatography–mass spectrometric method was developed. This method has also been successfully validated.

EXPERIMENTAL

Chemicals and reagents

Working standard of Tetrahydrobiopterin dihydrochloride (BH_4, Fig. 1) was obtained from Dipharma Caronno Pertusella Plant, Caronno Pertusella (VA) ITALY, and Metaxalone (Nirogi RV et al., 2006) (IS, Fig. 1) was procured from Clearsynth Labs (P) Ltd., Mumbai, India. Purity of both working standard was found to be >99%. HPLC grade acetonitrile and methanol were purchased from J.T.Baker (USA). LC-MS grade Ammonium Acetate was purchased from Sigma Aldrich (Fluka). HPLC grade Hydrochloric Acid was purchased from Rankem. Analytical grade Ascorbic acid was purchased from S.D.fine-chem limited. De-ionised water was produced from Milli-Q Gradient A-10 system (Millipore, MA, USA). Heparinised blank human plasma was collected from Reliance Life Sciences Private Limited, Navi Mumbai, India through ethical committee approved informed consent process.

HPLC Operating Conditions.

A Shimadzu (Shimadzu Scientific Instruments, Columbia, U.S.A.) Prominence LC system equipped with degasser (DGU-$20A_3$), pumps (LC-20AD) along with auto-sampler (SIL HTc) were used. 5µL aliquots of the processed samples were injected on a Hypurity Cyano 50x4.6mm, 5µm (Thermo Fisher Scientific Mumbai, India), which was kept at 25^{o}C temperature. The isocratic mobile phase, a mixture of Acetonitrile and 5mM Ammonium acetate Buffer Solution (65:35 v/v) was delivered at a flow rate of 800µL/min with a three way splitter into the mass spectrometer electro spray ionization chamber.

Mass spectrometry operating conditions

Quantitation was achieved by MS/MS detection in positive ion mode for analyte and IS using a MDS Sciex-Applied Biosystems (Foster City, CA, USA) API 4000 mass spectrometer, equipped with a Turboionspray™ interface at 500°C. The common source parameters like, curtain gas, nebulizer gas, auxillary gas and collision gas were set at 20, 30, 65 and 10 psi, respectively. The compounds parameters like, declustering potential (DP), collision energy (CE), collision exit potential (CXP) and entrance potential (EP) for BH4 and IS were 32, 26, 10, 8 Volts and 24, 18, 4, 11 Volts respectively. Detection of the m/z of analyte and IS was performed in the multiple reaction monitoring (MRM) mode and the MS/MS ion transitions were 241.9→ 166.0 for BH4 and 222.1→ 161.0 for IS. Quadrupole Q1 and Q3 were set on unit resolution. The analytical data was processed by Analyst software (version 1.4.1).

Preparation of stock and standard solutions

Working solutions of Calibration standards and quality control samples (QC) were prepared from two separate primary stock solutions of BH4. To prevent oxidation of BH4, the primary stock solutions (200.000 µg/mL) were prepared in 0.5% ascorbic acid in 0.1N HCl. Metaxalone stock solution of 200.000 µg/mL was prepared in methanol and diluted with Methanol:Milli-Q-Water (60:40) to prepare working internal standard (WIS) solution of 20.000 µg/mL. CC and QC stock solutions of BH4 were diluted with 0.5% ascorbic acid in 0.1N HCl to prepare working solutions of CC and QC samples respectively, to produce working solutions of 0.020, 0.040,0.100, 0.300, 0.500, 0.800, 1.200, 1.500, 2.000 µg/mL for CC and 0.060, 0.701, 1.402 µg/mL for QC. Working solutions were used to prepare plasma calibration standards. Calibration samples were prepared by freshly spiking 190µL of control human plasma with the appropriate working solution of the analyte (10µL) and 10µL of 20% ascorbic acid in 0.1 N HCl solution as a preservative was added in all

spiked samples and vortexed for complete mixing. All BH4 and MTX solutions were stored at -20°C. Samples for the determination of stabilities and effects were prepared by spiking control human plasma in bulk with BH4 at appropriate concentrations [1.000 (LLOQ), 3.005 (LQC), 35.056 (MQC) and 75.112 (HQC) ng/mL)] and 300 µL aliquots were distributed in different tubes. The samples were stored at -70 °C and -20°C.

Sample preparation

In case of fresh spiking (for preparing calibration standard and quality control samples) to 190 µL of plasma, 10µL of BH4 working solution and 10µL of 20% ascorbic acid in 0.1 N HCl Solution were added followed by vortexing. In case of bulk spiking (stability samples) 210µL of sample was aliquoted. To all the samples 10µL of WIS solution was added and vortexed for 30 seconds for complete mixing. 100µL of 5mM ammonium acetate buffer was added and vortexed. Then 1 mL of Acetonitrile was added and vortexed for 5 minutes. Samples were centrifuged at 15,000RPM for 5 minutes at 10^{o}C. Supernatant was transferred into HPLC vials for analysis.

Validation of the assay method

The validity of the assay method was assessed according to regulatory guidelines (US DHHS, FDA, CDER, 2001), with regard to the linearity, sensitivity, precision, accuracy, recovery and stability.

The QC samples at three concentrations (low, medium and high) were used for these tests. For construction of the standard calibration curve, working solutions of BH4 were spiked in to blank plasma to obtain concentrations of 1.000, 2.000, 5.000, 15.001, 25.002, 40.003, 60.004, 75.005 and 100.006 ng/mL. The between-run precision and accuracy of the method were evaluated at concentrations of 1.000, 3.005, 35.056 and 70.112 ng/mL. The within-run precision and accuracy of the method were evaluated at concentrations of 1.000, 3.005, 35.056, 70.112 and 100.006 ng/mL. To evaluate ruggedness, experiments were performed with a different analyst and different column.

Linearity and sensitivity

Calibration standards were prepared and analyzed at each concentration. Calibration curve was constructed using the analyte/IS peak area ratio versus the analyte's nominal concentration and fitted by linear least-squares regression analysis with weighting factor $1/x^2$. Sensitivity of the method was evaluated in terms of the lower limit of quantification (LLOQ). LLOQ was determined based on the two criteria: (1) the analyte response at the LLOQ should be at least 5 times the response compared to blank response and (2) analyte peak (response) should be identifiable, discrete, and reproducible with a precision within ≤20% and accuracy of 80–120%.

Precision and accuracy

Six replicates of QC samples were analyzed in the same run to determine within precision and accuracy, while in 4 separate runs QC samples were analyzed to determine the between-run precision and accuracy. Precision was calculated as the Coefficient of Variation (%CV), whereas accuracy was assessed as the percentage to the nominal concentration (%). The within and between-run %CV was set at ≤20% for QC samples at LLOQ concentration and ≤15% for other QC samples from respective nominal concentrations.

Recovery

Extraction recoveries of BH4 and IS were assessed by comparing the peak areas of the extracted QC samples to the post-extracted standard solutions containing equivalent amount of the analyte and IS. Briefly, 10µL of 20%Ascorbic acid in 0.1 N HCl solution and 10µL of 0.5%Ascorbic acid in 0.1 N HCl (Diluent for BH4) solution were added to 190µL of blank plasma and vortexed. Afterwards, 100µl of 5mM ammonium acetate buffer was added and vortexed. 1mL of Acetonitrile was added and vortexed. Centrifuged the samples at 15,000 rpm for 5 minutes at 10^{o}C. To 1080µL of supernatant, 10µL of QC working solution and 10µL of IS working solution were added and vortexed (post-extracted QC samples). The peak area, representing 100% recovery, was compared with that from the extracted QC samples.

Matrix effect

To determine the matrix effect, the post extracted samples of six blank matrices, including one sample each of Hemolysed and Lipemic at LQC level and aqueous LQC sample (six injection of aqueous sample) were analysed for assessing the lot to lot matrix effect. Post extracted samples were prepared at LQC level by spiking WLQC as well as WIS in the processed blank matrices. One aqueous LQC sample (pure solution of the Analyte) was prepared by spiking WLQC as well as WIS in the similar manner as of post extracted samples. Above processed post extracted samples and aqueous sample (six injection of aqueous sample) were analysed in a single

run. Similarly matrix factor (MF) of analyte and IS was calculated. The IS normalized MF was also calculated by dividing the MF of the analyte by the MF of the IS.

Stability

The solution stability (Long Term Stock and Working Solutions Stability) and auto-sampler stability of BH4 and MTX were evaluated. Stability was also evaluated for BH4 in Plasma samples (Coolant, long term in matrix, Bench Top at room temperature and freeze thaw stability). For freeze thaw stability assessment, QC samples were exposed to five freeze (-70°C) and thaw (at room temperature) cycles and then analyzed along with the freshly prepared samples. For Auto-Sampler Stability, the prepared samples in the auto-sampler were evaluated by analyzing the samples after being placed in the auto-sampler at 4°C for approximately 31 hours. For Bench Top Stability**,** the QC samples were prepared and kept at room temperature for approximately 06 hours and then analyzed along with the freshly prepared samples. For Coolant Stability, QC samples were prepared and kept under dry ice for approximately 70 hours and then analyzed along with the freshly prepared samples. All these stability tests were performed on LQC and HQC concentrations, each with six replicate samples. The samples were then processed and analyzed together with the freshly prepared samples. Long Term Stability of Analyte and Internal Standard in Stock and Working Solutions were evaluated by injecting vials, each from stability stock, comparison stock and working solution. Long Term Stability of Analyte in matrix was determined by analysing stability samples (six samples each of LQC and HQC stored at -20°C and -70°C freezer) along with the fresh calibration standards and six samples each of LQC and HQC (comparison samples). The percent change in concentration, over time, was used as an indicator of stability. The analyte was considered to be stable when the percent change was within ±15% of their respective nominal concentrations, In case of Stock and working solution stability, the analyte and Internal Standard were considered to be stable when the percent change was within ±10% of the nominal concentration.

Other experiments

Injector carryover and Dilution integrity tests were performed. For dilution integrity test, six replicates of the Diluted Quality Control (DQC-1.5*ULOQ concentration) were respectively diluted to 1/5th and 1/2nd time with human heparin plasma prior to extraction and analysis for evaluating dilution integrity. To evaluate the re-injection reproducibility, LQC and HQC samples of one P&A experiment were kept in auto-sampler after analysis at 4°C, and re-injected after 2 hours. Concentrations were calculated to determine % change after re-injection. Hemolysis and anticoagulant effects were performed to check the effect of hemolysis and different anticoagulant (other than heparin) on precision and accuracy of samples. One set of sample (six samples each of LQC and HQC) was prepared in hemolysed blank heparin plasma and another set was prepared in plasma containing EDTA as an anticoagulant. All samples were processed and analysed with freshly processed calibration standards in normal heparin plasma in a single run.

Whole blood stability of BH4 was evaluated at room temperature with and without addition of preservative. Working solution of BH4 was prepared at ULOQ using diluent Methanol:Milli-Q Water (60:40), containing no preservative. By spiking this working solution, two sets of samples (in duplicate) were prepared in fresh human heparin whole blood. At zero hour of drug spiking, equivalent volume of preservative was added in one set of blood samples. Both sets of samples (with and without preservative) were kept at room temperature for 30 minutes as a stability samples. To isolate the plasma, both sets of blood samples were centrifuged in a refrigerated centrifuge at 4°C with a rate of 3000 rpm for 10 minutes. Required quantity of preservative was added in the isolated plasma of another set of samples containing no preservative. All these stability samples were processed along with freshly processed set of calibration standards in normal heparin plasma and analysed in a single run.

RESULTS AND DISCUSSION

Mass spectrometry and chromatography

The full Q1 scan of BH4 and MTX were acquired in positive ion mode by infusing the standard solutions of 100.000 ng/mL concentration, prepared in Mobile phase and 60% methanol in water respectively, into ESI source. The Product ion mass spectra of these two compounds are shown in Fig. 2. The most abundant product ion of each analyte was selected for MRM monitoring, and the MS/MS conditions were optimized to maximize the response of each of precursor/product transition (Table 1).

Chromatography and specificity

Representative chromatograms of Plasma Blank, Zero Standard, Lower Limit of

Quantitation, Upper Limit of Quantitation along with IS are displayed in Figure 3. The typical retention time was 1.05min for BH4 and 0.96min for MTX with a total run time of 7.0min. These result shows that BH4 at high and low concentrations can be clearly detected. No significant interfering peaks at the retention time of BH4 and the IS were observed, which shows that there was a lack of interference observed (from the endogenous components in plasma) at the corresponding peaks of BH4 and IS. No significant injector carry over was observed at the RT of both Analyte and IS.

Linearity, sensitivity, precision and accuracy

The calibration curve of BH4 was linear over the concentration range of 1.000-100.006 ng/mL and is shown in Figure 4. The correlation coefficient was greater than 0.9990 for multiple analytical runs. The best-fit line of the calibration curve was obtained by using a weighting factor of $1/x^2$. The mean linear regression equations (n = 4) were: Y = 0.011125 (±0.0007) ×0.002630 (±0.0007), (r = 0.9994±0.0004). The LLOQ was 1.000 ng/mL.

The precision and accuracy of the assay method are summarized in Table 2. For QC samples, the within run coefficient of variations ranged from 1.59 to 6.47%, while the between run coefficient of variations ranged from 5.48 to 9.52%. The accuracy (presented as percent deviation from the nominal concentrations) ranged from 89.55 to 99.41. The method was found to be rugged with different analyst, and with different column of same make and specifications. These results show that this method is accurate, precise and reproducible for estimation of BH4 in human plasma.

Recovery and matrix effect

Recovery of BH4 was evaluated by comparing mean analyte area response of six extracted samples of low, medium and high quality control samples to mean analyte area response of six un-extracted (samples prepared in extracted plasma blank) samples of low, medium and high quality control samples.

This method yielded a recovery of 75.46% to 85.20% for BH4 and 91.66% for MTX which shows that recovery rate was consistent over the calibration range. No effect of matrix (six different lots of heparin plasma including one sample each of Hemolysed and Lipemic) was observed on analyte quantitation. The Mean Matrix Factor for BH4 and Metaxalone was 0.45 and 0.81 respectively. The mean Internal standard Normalized Matrix Factor was 0.56. The coefficient of variation of Internal standard Normalized Matrix Factor was 3.74%. During method development it was observed that low matrix factor can be resolved by using long analytical column and by increasing percentage of buffer in the mobile phase composition, but it leads to long run time. Matrix factor can also be improved by using special types of SPE cartridges (SUPELCO, Hybrid SPE® phospholipid 30mg/1mL).

Stability

The BH4 was found to be stable in plasma under the storage conditions (-70°C) for at least 30 days, and room temperature for approximately 06 hours which is long enough to cover the whole study duration. Upon five freeze thaw cycles, almost no difference was observed in the peak areas of QC samples in comparison to the freshly prepared samples. No significant degradation of BH4 and IS was observed when the extracted samples were kept in the auto-sampler at 4°C temperature for approximately 31 hours. BH4 was found stable under dry ice for approximately 70 hours. For both BH4 and Metaxalone, stock and working solutions were found to be stable for 16 days. The obtained concentrations for BH4 at 3.005 and 70.112 ng/mL were within ±15% of the respective nominal concentrations in a series of stability tests like, coolant (70 h), repeated five freeze thaw cycles, bench-top (06 h), auto-sampler (31 h) and long term freezer stability at temperatures of -70°C & -20°C for at least for 30 days (Table 3). The results were found to be within the assay variability limits during the entire process.

Other experiments

No significant Injector carryover was observed. The results have shown that the precision and accuracy of diluted samples were within acceptable range. BH4 was found to be stable in auto-sampler at 4°C and reproducible after re-injection. No effect of hemolysis and anticoagulant was observed on analyte quantitation. Furthermore it was observed that BH4 is stable at room temperature for 30 minutes in human heparin whole blood only after addition of equivalent volume of preservative.

CONCLUSION

The present LC–MS/MS method provides a direct, simple, robust, fast and sensitive analytical tool for BH4 in human plasma and can be successfully applied to clinical pharmacokinetic study in human subjects. To prevent oxidation of BH4 in human heparin

whole blood samples collected during pharmacokinetic, bioavailability and bioequivalence study, all these samples should immediately be mixed with equivalent volume of preservative after their sampling; and resultant plasma must be isolated within 30 minutes of their respective collection time.

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Table 1: MS conditions for BH4 and IS

Drug Name	Parent mass (Q1) $[M+H]^+$	Product mass (Q3)	Time (msec.)	DP(V)	EP(V)	CE(V)	CXP(V)
BH4	241.9	166.0	400	32	8	26	10
MTX	222.1	161.0	400	24	11	18	4

DP, declustering potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy; CXP, collision cell exit potential.

Table 2: Within and Between-Run precision of determination of BH4 in human plasma

Added concentration (ng/mL)	Mean ± SD calculated concentration (ng/mL)	CV (%)	Accuracy (%)
Within-Run: n=6(Six replicates at each concentration)			
1.000	0.994±0.0643	6.47	99.41
3.005	2.793±0.0996	3.57	92.94
35.056	32.582±1.0071	3.09	92.94
70.112	63.775±1.0120	1.59	90.96
100.006	89.558±3.6614	6.47	89.55
Between-Run n=24 (Twenty four replicates at each concentration)			
1.000	0.951± 0.0905	9.52	95.08
3.005	2.874± 0.2455	8.54	95.65
35.056	32.648± 1.7894	5.48	93.13
70.112	65.716± 5.4682	8.32	93.73

Table 3:Stability data of BH4 quality controls in human plasma

Nominal concentration (ng/mL)	Stability	Mean ± SDa n = 6 (ng/mL)	Precision (% CV)	Change (%)b
3.005	70 h (coolant)	3.009 ± 0.1421	4.72	-9.94
	Fifth freeze thaw	3.070 ± 0.0868	2.83	-8.10
	06 h (bench-top)	2.955 ± 0.2559	8.66	-3.58
	20 h (auto sampler)	2.791 ± 0.1806	6.47	-4.51
	Thirty day at -70°C	2.751± 0.4356	10.72	-5.90
	Thirty day at -20°C	2.793 ± 0.0996	4.72	-4.46
70.112	70 h (coolant)	72.521 ± 2.3601	3.25	-3.42
	Fifth freeze thaw	72.859 ± 1.9555	2.68	-2.97
	06 h (bench-top)	70.531± 2.7476	3.90	-6.09
	20 h (auto sampler)	65.180 ± 2.9787	4.57	-0.27
	Thirty day at -70°C	66.571± 4.2671	6.41	1.86
	Thirty day at -20°C	63.775± 1.0120	1.59	-2.42

[a] Back-calculated plasma concentrations

[b] With respect to comparison sample concentration.

Legends to Figures

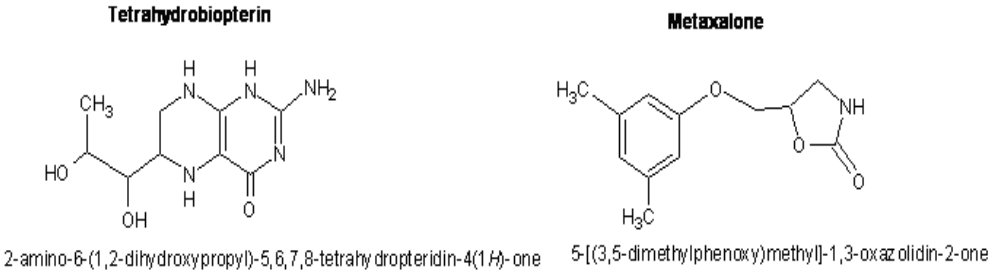

Fig. 1: Structural representation of BH4 and MTX

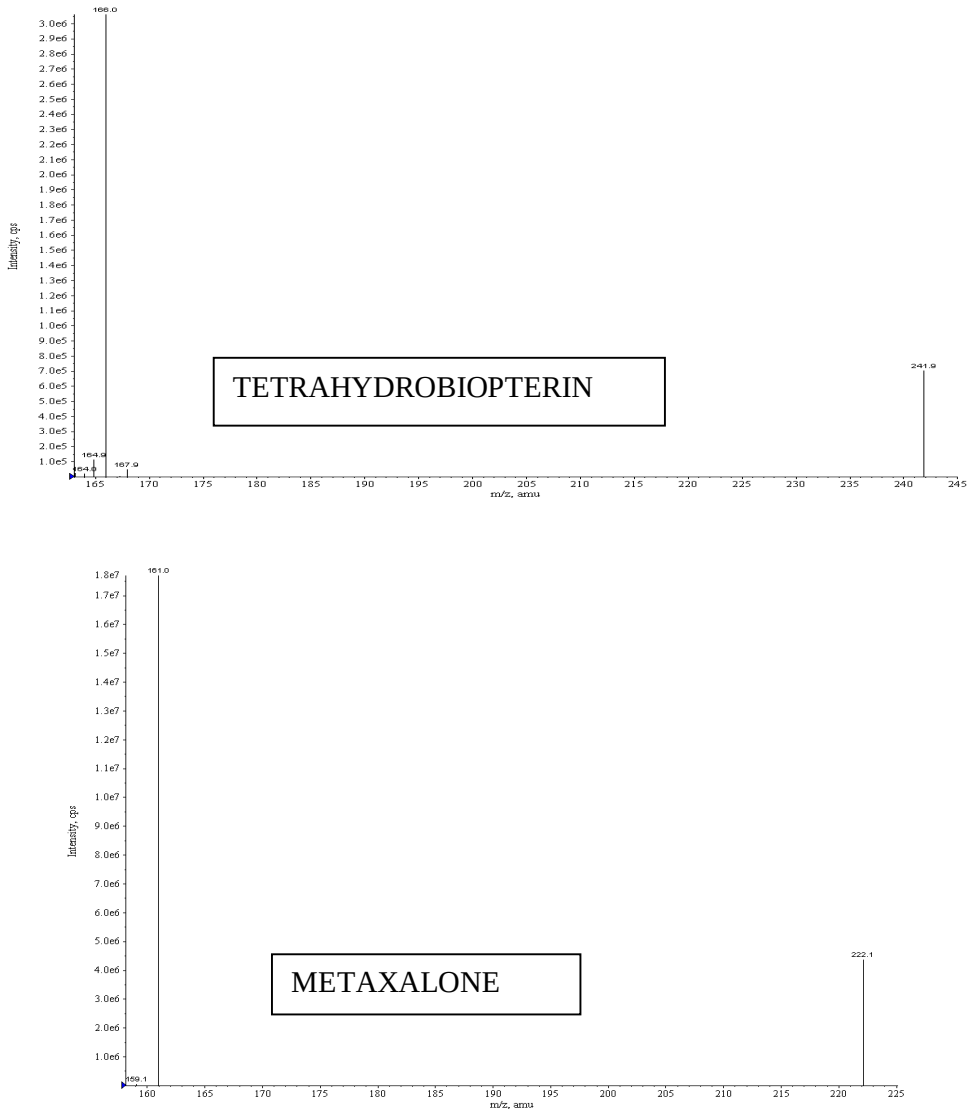

Fig. 2: Product ion mass spectra of BH4 and MTX

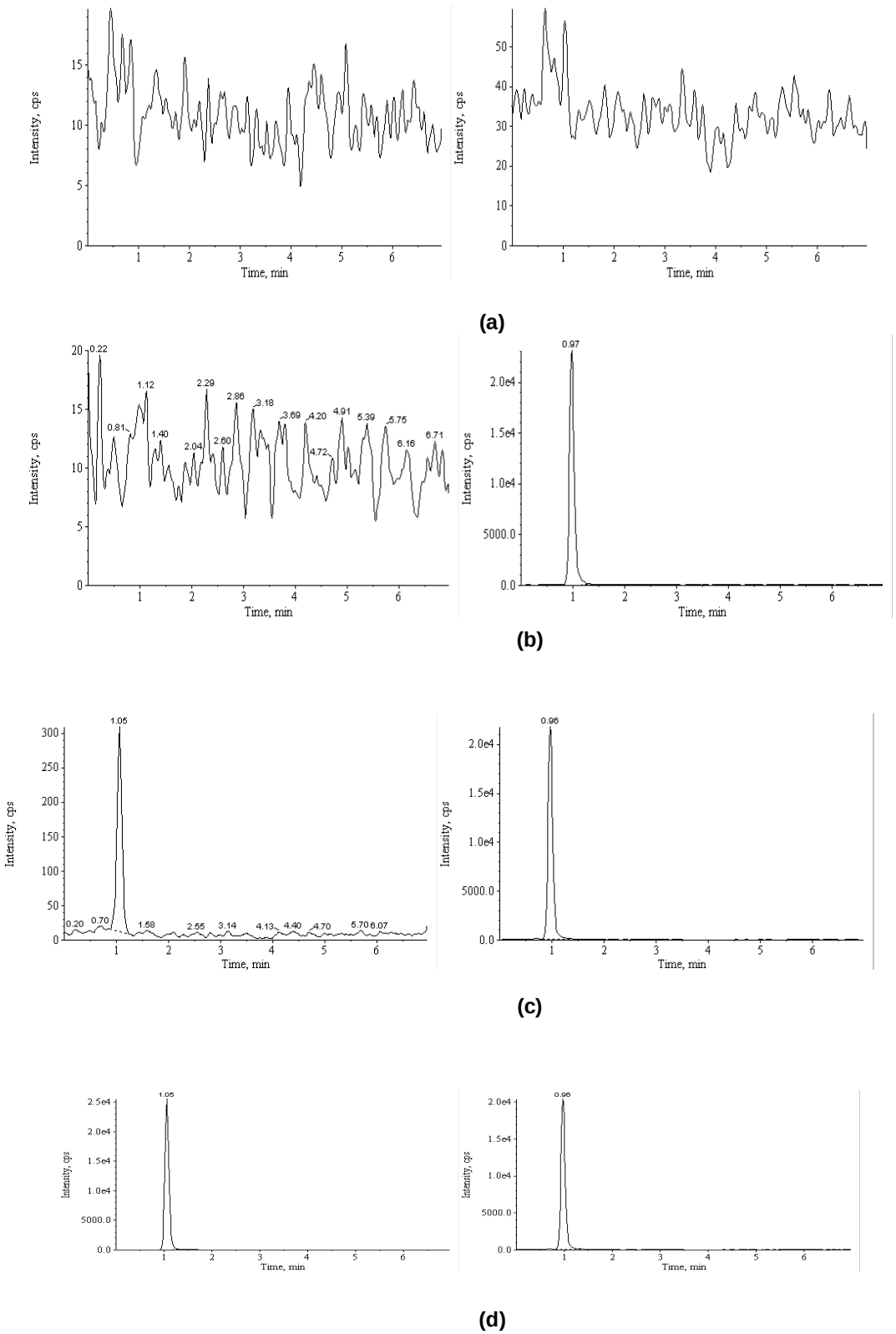

Fig. 3: Typical MRM chromatograms of BH4 (left panel) and IS (right panel) in human blank plasma (b) human plasma spiked with IS (c) human plasma spiked with BH4 at LLOQ (1.000 ng/mL) and IS and (d) human plasma spiked with BH4 at ULOQ (100.006ng/mL)

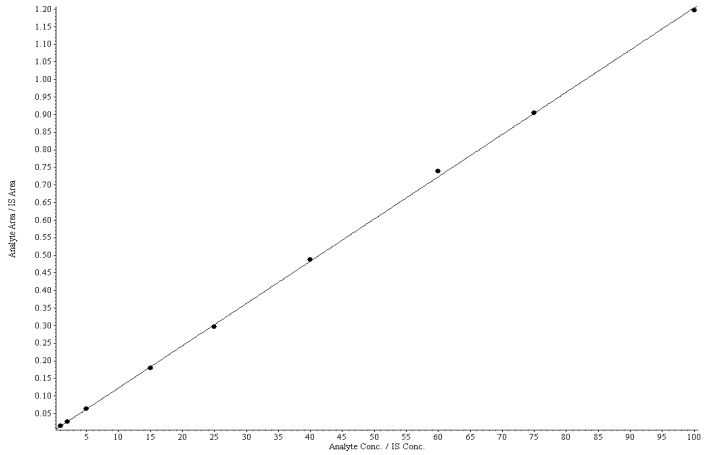

Fig. 4: Calibration curve of BH4

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