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Journal of Chromatography B, 803 (2004) 311–315
JOURNAL OF CHROMATOGRAPHY B
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Determination of derivatized L-alanosine in plasma by liquid chromatography-tandem mass spectrometry

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Received 20 November 2003; received in revised form 5 January 2004; accepted 7 January 2004

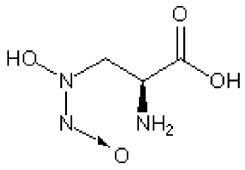
Abstract

A sensitive method was developed for quantitation of the cytotoxic antibiotic L-alanosine in human plasma. Alanosine was extracted from plasma by anion-exchange solid phase extraction, derivatized with dansyl chloride and analyzed by liquid chromatography-tandem mass spectrometry using atmospheric pressure chemical ionization in negative mode. Dansylation led to 50-fold improvement of method sensitivity over non-dansylated alanosine with a resulting 20 ng/ml limit of alanosine quantitation in plasma being achieved. The method was validated and applied for clinical studies of alanosine administered to cancer patients.

Keywords: Derivatization, LC; Alanosine

1. Introduction

L-Alanosine is an antibiotic derived from *Streptomyces alanosinincus*. This novel amino acid possesses antitumour



L-Alanosine. Mol.wt.149.1

properties [1]. It has been suggested that the positive response of cancer patients to alanosine treatment is related to alanosine selective toxicity for tumor cells deficient in methylthioadenosine phosphorylase (MTAP). MTAP is an important enzyme required for the synthesis of adenosine monophosphate (AMP) via a purine salvage pathway. In the face of a dysfunctional purine salvage pathway, MTAP deleted tumors are becoming completely dependant on de-novo synthesis of AMP, a biosynthetic pathway blocked by L-alanosine. Encouraging preliminary clinical results led to the premise that patients with MTAP-deficient tumors could preferentially be treated with L-alanosine [2,3]. Alanosine apparently has a short half-life in plasma. Only a few minutes after intravenous administration of radiolabled alanosine to mice, metabolites accounted for 85% of total radioactivity [4]. The low molecular weight of alanosine combined with its pronounced hydrophilic properties result in poor reverse-phase chromatographic separation and detectability. This explains the dearth of published in the last twenty five years analytical methods describing alanosine quantitation in biological matrices. A literature survey revealed only one publication in which the authors described a method using dansyl chloride (DNS) derivatization of alanosine in plasma followed by HPLC analysis with fluorescence detection. The limit of detection for this method was 100 ng/ml [5]. DNS is a well known derivatization reagent for amino groups in various molecules [6–8]. Most DNS applications employ fluorescence detection. The possibility of alanosine detection by mass spectrometry (MS) had not yet been explored. The objective of this research was to develop a reliable method of L-alanosine quantitation in human plasma achieving low limit of quantitation in the 10–20 ng/ml range.

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doi:10.1016/j.jchromb.2004.01.003

2. Experimental

2.1. Method development

It was initially hoped that the simple substitution of fluorescence detection by more sensitive MS detection would give us the desired 10-fold gain in sensitivity. Preliminary experimentation with underivatized alanosine, however, did not allow descent below 1 μg/ml in method sensitivity. Several derivatization compounds, such as *o*-phthalaldehyde, pentafluorobenzyl bromide, bis(trimethylsilyl) trifluoroacetamide and DNS (molecular weight 269.8) were tested for potential bonding with amino, hydroxyl or carboxylic groups in alanosine molecule. Each of tested derivatization agents exhibited very high noise levels in full MS scan. The highest yield and stability of a derivatizied product were obtained with DNS. A single derivatization of alanosine molecule with the loss of two hydrogen and one chlorine ion led to the occurrence of 381 *m/z* ion. This ion was unfortunately among the least prominent in the derivatization mixture (Fig. 1). Dually-derivatized alanosine (ion 614 *m/z*) has also occurred under these reaction conditions (refer to Section 2.2) but with considerably lower yield than 381 *m/z* ion. The relative abundance of single- and dually-derivatized ions was approximately 6:1. Variations of temperature, derivatization time and pH (range of 7–9) had very little effect on the derivatization product ratio. Increasing the pH of the derivatization solution above 9 was avoided to prevent DNS spontaneous hydrolyses. Presence of ammonia ions was determined to interfere with alanosine–DNS bond formation and was eliminated during derivatization. The original HPLC method [5] required alanosine–DNS derivatization before extraction from plasma due to binding of non-derivatized alanosine to plasma proteins in the precipitation step. An attempt to follow the lengthy published procedure, which utilized freeze-drying of samples, resulted in low recovery and poor accuracy. Derivatization after alanosine extraction from plasma was selected as an alternative method. Cation-exchange extraction of alanosine from plasma (10 mg MCX plate from Waters) showed greater than 50% recovery but high matrix interference, possibly due to co-elution of other plasma amino acids. The presence of endogenous amino acids in the extracted samples might also have been the reason for high MS interference level with ^{15}N-threonine and ^{15}N-phenylalanine tested as possible internal standards. Anion-exchange using 10 mg MAX plate (Waters) yielded approximately 25% alanosine recovery and good specificity and was chosen for sample clean up, to be followed thereafter by derivatization. ^{15}N-threonine and ^{15}N-phenylalanine tested as potential internal standards showed no recovery under anion-exchange conditions. Alanosine recovery from plasma using the MAX plate was evaluated within the pH-range of 7–10. The highest recovery was obtained in the 9–10 pH-range (Table 1). Mixing human plasma with an equivalent amount of 0.02 M sodium hydroxide provided the desirable pH of approximately 9.5. Alanosine recovery from plasma dropped considerably at pH below 9.

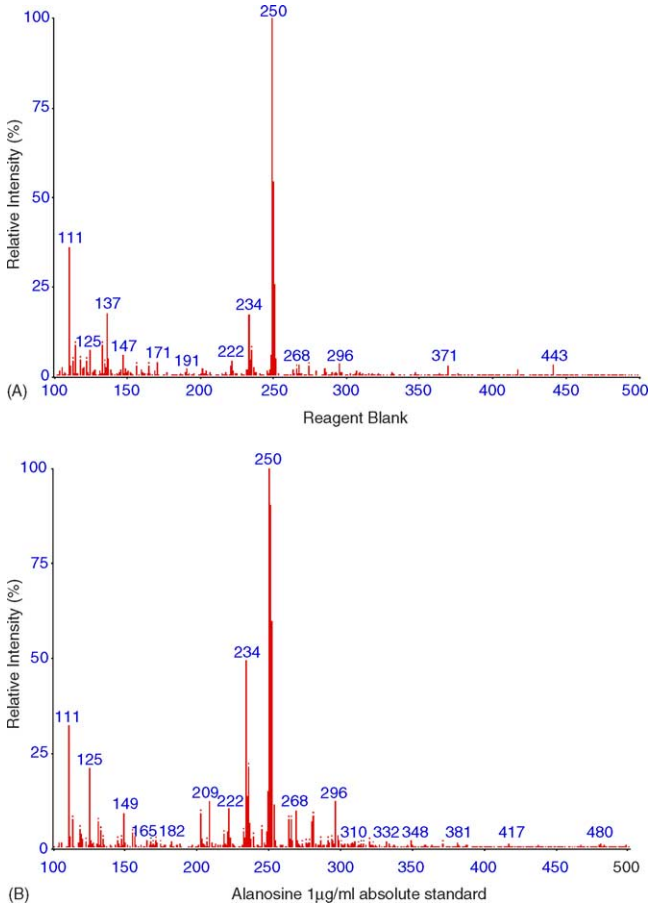

Fig. 1. L-Alanosine derivatized with DNS. Q1 scan in 100–500 *m/z* range: (A) reagent blank and (B) alanosine 1 μg/ml absolute standard.

2.2. Method procedure

Analytical grade methanol, acetonitrile, acetic acid, ammonium acetate, sodium dihydrogen phosphate, sodium hydroxide and acetone were supplied by VWR (Canada). Blank human plasma anti-coagulated with Na_2EDTA was purchased from VWR (Canada). DNS was purchased from Sigma–Aldrich (USA). L-Alanosine standard

Table 1
The pH effect on alanosine recovery from human plasma

Plasma pH	L-Alanosine spiked concentration (ng/ml)	Peak area counts (ion 381.2 → 250.1)
7	200	Not detected
8	200	1524
9	200	3540
10	200	3856
7	2000	18203
8	2000	39592
9	2000	66507
10	2000	68364

(3-[hydroxynitrosoamino]-L-alanine) was provided by Pharmaceutical Development Center (USA). The alanosine isotope D,L-alanosine-$^{15}N_2$ (3-[hydroxy-^{15}N-nitroso-^{15}N-amino]-D,L-alanine) used as internal standard (IS) was synthesized by Toronto Research Chemicals Inc. (Canada) specifically for this project. Anion-exchange 96-well extraction plates (10 mg MAX, "Oasis") were purchased from Waters (USA). To obtain a 1 mg/ml stock solution, neat L-alanosine standard was first dissolved in 0.01 M sodium hydroxide by sonication and then further diluted with methanol to achieve the ratio 80:20 (v/v) of 0.01 M sodium hydroxide:methanol. D,L-Alanosine-$^{15}N_2$ internal standard stock solution was prepared in the identical fashion. Stock standards have been shown to be stable for at least 2 months when stored at −20 °C. Further dilutions of L-alanosine standard stock solution were made in methanol. IS stock solution was diluted with 0.02 M sodium hydroxide to obtain a concentration of 50 ng/ml. A 0.01 M dansyl chloride solution was prepared in acetone on a biweekly basis and stored at −20 °C. Derivatized absolute standards were stable for at least one month when stored at −20 °C.

Method validation was conducted under GLP conditions as per FDA guidance [9]. Calibrators and QC samples were prepared by spiking human plasma with L-alanosine standards made in methanol. A 100 μl aliquot of spiked plasma sample was mixed with 100 μl of IS (50 ng/ml) made in 0.02 M sodium hydroxide. Blank plasma samples were mixed with an equal amount of 0.02 M sodium hydroxide omitting IS. MAX plate was conditioned with 200 μl of methanol followed by 200 μl of 0.01 M phosphate buffer (pH = 9.5). A 150 μl aliquot of plasma mixed with IS was applied to the plate. After washing of the retained sample with 200 μl methanol, samples were eluted into a 96-cell liquid chromatography (LC) block with 600 μl of 10% acetic acid in methanol (v/v). Samples were dried under nitrogen on a block dryer (SPE Dry-96, Jones Chromatography) at approximately 50 °C and reconstituted in 100 μl methanol. A 25 μl volume of 0.01 M phosphate buffer (pH = 8.0) and 25 μl of 0.01 M DNS in acetone was added to each cell and sealed with a plastic cover. The sample block was placed at 50 °C for 40 min to allow derivatization. Following derivatization, samples were dried at approximately 50 °C as stated above and reconstituted in 100 μl of 70:30 (v/v) mixture of acetonitrile: 25 mM ammonium acetate in 0.5% acetic acid.

The LC–MS–MS system was comprised of an Agilent 1100 binary pump with CTC Analytics PAL autosampler and Sciex API III+ triple quadrupole MS detector. An Agilent Eclipse XDB-C8, 4.6 mm × 150 mm, 5 μm column was used. The LC-pump channel "A" contained 25 mM ammonium acetate in 0.5% aqueous acetic acid, while channel "B" contained acetonitrile. A mobile phase flow rate was 0.7 ml/min. The following mobile phase gradient was applied: initial 70% flow from channel "A" linearly changed to 30% at 2.2 min, held for 1.7 min, instantly changed to initial condition and re-equilibrated for 1 min. Retention time for alanosine and its IS was approximately 3.5 min. Run time was 5 min, injection volume was 30 μl, column temperature was ambient (nominally 25 °C) and needle wash solution was 50:50 methanol:water. MS detection was performed by atmospheric pressure chemical ionization in the negative ion

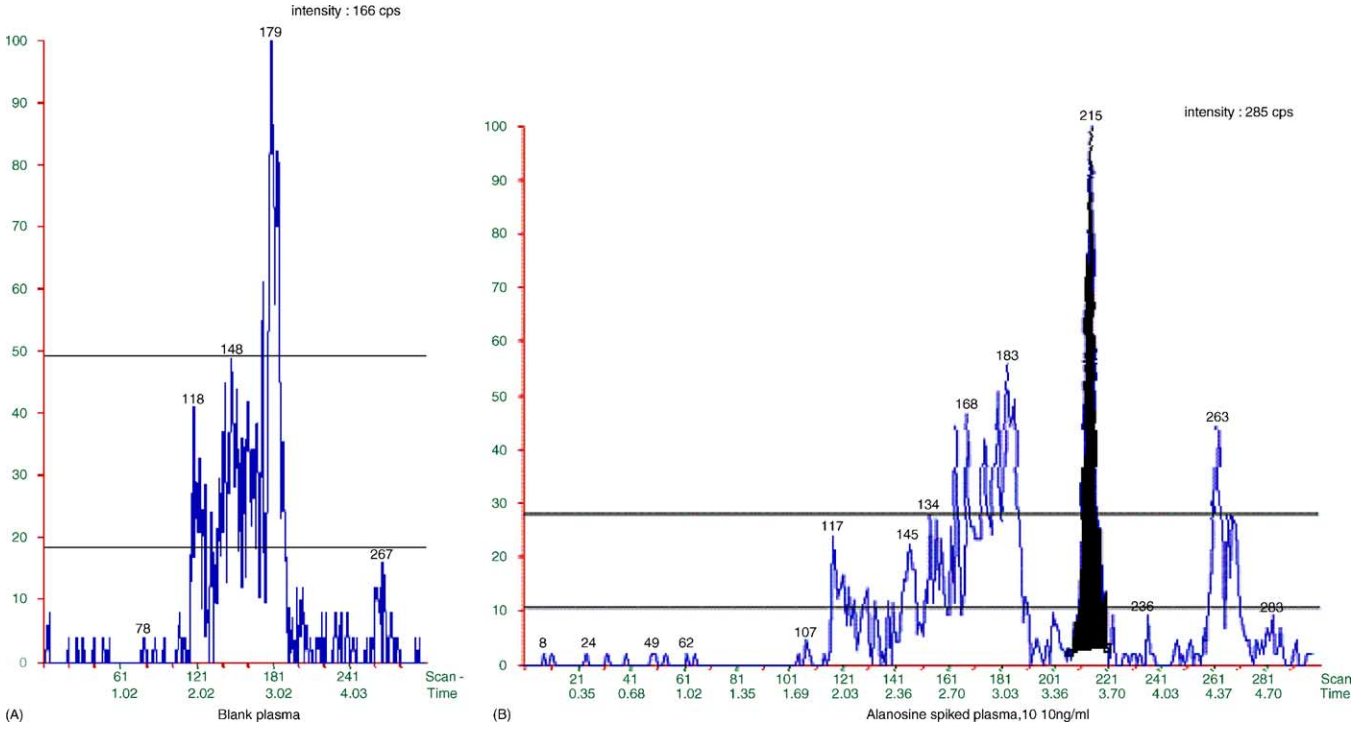

Fig. 2. Chromatograms of blank plasma (A) and 10 ng/ml alanosine spiked plasma (B). Signal obtained in MRM (381.2 → 250.1).

mode. Nebulizer temperature was 500 °C, interface temperature was 50 °C. Ultra dry nitrogen was used as a carrier and curtain gas with argon as a collision gas. A 381.2 *m/z* ion was selected as the precursor ion for derivatized alanosine and a 383.2 *m/z* ion was selected for the IS. Both standards had the same product ion 250.1 *m/z*. The data were acquired in the multiple reaction monitoring mode: 381.2 → 250.1 for L-alanosine and 383.2 → 250.1 for D,L-alanosine-$^{15}N_2$. The dwell time was 300 ms with a 200 ms pause.

2.3. Method validation

The possibility of achieving 10 ng/ml low limit of quantitation (LOQ) was investigated. At this level, interference from the blank matrix is substantial (Fig. 2), and although gradient elution had helped to separate interference peaks from the peak of interest, 10 ng/ml could not be quantified reliably. Method sensitivity was also examined at 20 ng/ml. Based on the accuracy calculations at the 10 and 20 ng/ml levels (Table 2), LOQ was determined to be 20 ng/ml. Cross interference of L-alanosine and D,L-alanosine-$^{15}N_2$ product ions in MRM mode prevented us from extending the upper limit of quantitation beyond 400 ng/ml. The parameters tested for method validation included linear range, method precision, accuracy, sensitivity, specificity and stability. Method validation was conducted in the linear range of 20–400 ng/ml (Fig. 3). Method precision and accuracy throughout the linear range of the method was demonstrated by assaying three quality control (QC) levels (60, 250 and 350 ng/ml) at six replicates on each of 4 days. Although inaccuracy exceeded 15% value in two sets (Table 3), four out of six (67%) QC samples at these sets remained within ±15% inaccuracy acceptance criteria. No deviation from target values was observed when low and high QC levels were spiked into six different sources of human plasma, demonstrating the specificity of the method. Room temperature stability of alanosine in human plasma was verified over 48 h by testing six replicates at each of the three QC levels. Maximum change in reported concentration when compared to Time 0 was 8.5% (Table 4). Alanosine stability in plasma over 48 h may be an indication that the short half-life in plasma reported by several authors [4,5] is related to fast clearance from plasma rather than enzyme degradation of alanosine in plasma.

Table 2
Method sensitivity comparison at 10 and 20 ng/ml L-alanosine in human plasma

	10 ng/ml		20 ng/ml	
	Calculated value	Signal/noise	Calculated value	Signal/noise
	2.5	12.0	19.4	29.4
	8.3	16.1	14.8	22.1
	9.4	17.1	21.5	25.7
	2.7	11.6	19.6	30.1
	8.2	13.7	18.9	25.2
	6.1	17.1	17.2	39.2
Mean	6.2	14.6	18.6	28.6
S.D.	3.00	2.50	2.32	6.0
R.S.D. (%)	48.5	17.1	12.5	20.8
Inaccuracy (%)	−38.3		−7.0	

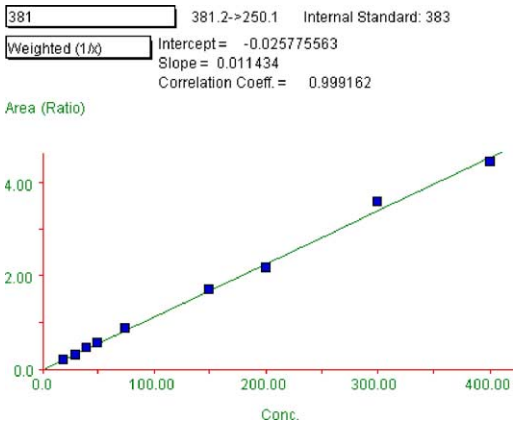

Fig. 3. Calibration curve obtained in the concentration range 20–400 ng/ml.

Table 3
Accuracy and precision of quality control samples

Concentration (ng/ml)	Day 1	Day 2	Day 3	Day 4
60.0	61.6	73.7	62.0	67.5
	55.7	73.7	61.5	64.8
	63.3	63.1	62.0	66.6
	65.9	74.1	67.8	78.8
	58.8	77.5	60.3	80.2
	[a]	58.3	59.0	60.1
Mean	61.1	70.1	62.1	69.7
S.D.	3.94	7.55	3.01	8.02
R.S.D. (%)	6.5	10.8	4.9	11.5
Inaccuracy (%)	1.8	16.8	3.5	16.1
250.0	274	267	257	281
	255	227	258	278
	262	238	255	280
	275	261	266	262
	272	266	263	273
	272	220	243	282
Mean	268	246	257	276
S.D.	7.81	20.7	7.83	7.52
R.S.D. (%)	2.9	8.4	3.0	2.7
Inaccuracy (%)	7.3	−1.4	2.8	10.5
350.0	382	350	350	390
	386	334	381	382
	358	395	333	382
	346	384	299	410
	372	383	357	447
	332	349	367	396
Mean	363	366	348	401
S.D.	21.2	24.7	28.8	25.1
R.S.D. (%)	5.8	6.8	8.3	6.3
Inaccuracy (%)	3.7	4.5	−0.6	14.6

[a] Data loss due to a computing error.

Table 4
Stability of alanosine in human plasma at room temperature

Concentration (ng/ml)	Time 0	24 h	48 h
60			
Mean	69.7	68.3	69.8
S.D.	8.05	5.02	3.57
R.S.D. (%)	11.5	7.4	5.1
Percent difference from time 0	–	−2.0	0.2
250			
Mean	276	273	262
S.D.	7.56	18.5	15.1
R.S.D. (%)	2.7	6.8	5.8
Percent difference from time 0	–	−1.3	−5.0
350			
Mean	401	380	367
S.D.	24.8	22.5	17.6
R.S.D. (%)	6.2	5.9	4.8
Percent difference from time 0	–	−5.3	−8.5

3. Conclusion

The method validation demonstrated good accuracy and precision of alanosine quantitaton in human plasma despite the potential for variability introduced by derivatization. An important element contributing to the method accuracy is the use of isotope-marked alanosine as internal standard. In addition to compensating for any recovery variations, such an IS has matching derivatization characteristics when compared with the analyte of interest permitting for ideal control over derivatization variables. The developed method allowed a five-fold sensitivity improvement over the published method of dansylated alanosine fluorescence detection [5]. The extraction procedure was also simplified with the gain in accuracy due to the internal standard use. The established detection limit of 20 ng/ml is a 50-fold improvement over MS detection of non-derivatized alanosine. Similar observations of MS detection enhancement upon dansylation was made for several polar urinary metabolites, derivatization of which led to the formation of more hydrophobic, readily ionizable compounds that otherwise were undetectable in MS with ion spray mode [10].

The robust capabilities of Sciex API III+ MS detector do not seem to limit the achieved method sensitivity. In a recent study we have reported sensitivity of 10 pg/ml for chloramphenicol on the same instrument under similar detection conditions [11]. Transferring the alanosine method to the more powerful API Sciex 4000 MS detector did not further improve the alanosine detection limit. Perhaps alanosine derivatization and ionization yields are the key limiting factors in alanosine MS detection sensitivity.

Acknowledgements

The authors would like to thank Teresa Sanelli, Craig Franklin, Mike Leadley and Jason Brittain for the technical expertise they contributed to this work.

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