RSC Advances

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. This *Accepted Manuscript* will be replaced by the edited, formatted and paginated article as soon as this is available.

You can find more information about *Accepted Manuscripts* in the **Information for Authors**.

Bioavailability, plasma protein binding and metabolic stability studies of ALDH2 activator, alda-1, using validated LC-ESI-MS/MS method in rat plasma

Isha Taneja*[1,2], Kanumuri Siva Rama Raju*[1,2], Monika Mittal[3], Kapil Dev[1,4], Mohammad Faheem Khan[1,4], Rakesh Maurya[1,4], Muhammad Wahajuddin[1,2]

[1] Academy of Scientific and Innovative Research, New Delhi, India. [2] Pharmacokinetics and Metabolism Division, CSIR- Central Drug Research Institute, Lucknow, India. [3] Division of Endocrinology, CSIR- Central Drug Research Institute, Lucknow, India. [4]Medicinal and Process Chemistry Division CSIR- Central Drug Research Institute, Lucknow, India.

*Equally contributing authors

Corresponding author:

Dr. Wahajuddin,

Scientist, CSIR-Central Drug Research Institute, Lucknow-226031, Uttar Pradesh, India

Tel.: +91-522-2772450/2550 (Ext-4849/50); fax: +91-522-2771941

E-mail address: wahajuddin@cdri.res.in / wahajuddin@gmail.com

Abstract:

Alda-1 is an activator of the enzyme ALDH2. It has been suggested as a novel therapeutic for cardiovascular implications such as myocardial infarction, coronary bypass surgery, heart transplantation, peripheral artery disease, ischemia reperfusion injury, angina and alcoholic cardiomyopathy. Despite its widespread experimental use, no reports are available on its pharmacokinetics or bioanalytical quantification. In the present study, a simple, precise and reliable LC-ESI-MS/MS method has been developed and validated for the first time for quantification of alda-1 in plasma. Alda-1 was analyzed on a C18 column using methanol and 0.1% formic acid (60:40) v/v as mobile phase at a flow rate of 0.7 mL/min. The method was found to be linear within the concentration range of 1-500 ng/mL. The intra- and inter-day precision and accuracy were within acceptable limits. For the first time, the preclinical oral and intravenous pharmacokinetics of alda-1were conducted. Alda-1 was found to be a rapidly absorbed, high clearance and poorly bioavailable compound in rats. Its plasma protein binding was found to be 82-86 %. In view of the new regulatory guidelines, incurred sample reanalysis was also performed and all the samples were found within 15 % of mean value. From the in *vitro* microsomal incubation studies, it was found to be a high extraction compound. The data presented here provide important information to support the *in vivo* efficacy of alda-1 and would be helpful in its further development as a therapeutic agent and synthesis of its analogs with better systemic exposure and disposition properties.

Introduction:

Aldehyde dehydrogenases (ALDH) are a group of enzymes catalyzing $NAD(P)^+$ dependent oxidation of aldehydes. The two most important isoforms responsible for converting aldehydes into non-toxic acids are its cytosolic form, ALDH1, and its mitochondrial form, ALDH2. The mitochondrial enzyme is the second enzyme in the oxidative detoxification of ethanol. It is also involved in the metabolism of toxic endogenous aldehydes such as 4-hydroxynonenal (4-HNE) and environmental aldehydic pollutants such as acrolein as well as in the bioconversion of nitroglycerin. Additionally, ALDH2 has also been implicated to play a cardio-protective role in ischemia induced heart damage during myocardial infarction and an inverse correlation has been suggested between ALDH2 activity and infarct size [1-2].

In approximately 40% of the East Asians, ALDH2 occurs as mutant ALDH2*2 due to a single nucleotide substitution of G to A in exon 12, resulting in glutamate at position 487 instead of lysine. This single amino acid polymorphism causes disruption of the co-enzyme NAD binding thereby leading to reduced catalytic activity. The mutant ALDH2*2 has 200 fold higher K_m for NAD^+ and 10 fold lower k_{cat} as compared to the wild-type enzyme [3]. As a result, the homozygous ALDH2*2 allele have 1-5% activity of the wild-type ALDH2*1 while the heterozygous ALDH2*1/*2 have 10-45% activity. In individuals having mutant ALDH2, acetaldehyde accumulates upon consumption of alcohol causes Asian flushing syndrome/alcohol flush reaction, characterized by facial flushing, nausea, drowsiness and increased heartbeat. The mutant ALDH2 also leads to decreased vasodilatory effect of nitroglycerin used in the treatment of angina, hypertension and myocardial infarction. An increased susceptibility to upper digestive tract cancers and neurodegenerative diseases has also been associated with ALDH2*2.

Alda-1, chemically *N*-(1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide, is an activator of the enzyme ALDH2, acting as a structural chaperone and restoring the activity of ALDH2*2 [1]. It has been proposed that Alda-1 promotes the apparent binding affinity for NAD^+ the essential cofactor for the functioning of ALDH2. Thus, it reinstates the structural and functional conformation of the active site of ALDH2 resulting in its increased dehydrogenase, esterase and nitroglycerin denitration activity [4]. The binding of alda-1 decreases the apparent K_m for NAD^+ by 6.7-fold and increases the V_{max} by 2-fold [3]. Due to the protective role of ALDH2 in regulation of free radical generation, metabolism of 4-HNE and mitochondrial dysfunction, activators of ALDH2 such as Alda-1 that restore its activity have been advocated as novel therapeutics for cardiovascular implications such as myocardial

infarction, coronary bypass surgery, heart transplantation, peripheral artery disease, ischemia reperfusion injury, angina and alcoholic cardiomyopathy [5-6]. The *in vivo* cardio-protective role of alda-1 has been confirmed in myocardial infarction rat model. Administration of alda-1 at 8 mg/kg prior to ischemia reduced the infarct size by 60% [2]. Despite the progressive research on its clinical utility and its popular use as an experimental tool, no literature is reported describing its pharmacokinetic and disposition aspects. Also, to the best of our knowledge, no method is reported for its quantification in biomatrix. Thus, in the present study, we have developed and validated an LC-ESI-MS/MS method for the quantification of alda-1 in rat plasma for the first time. We have also determined its *in vivo* preclinical pharmacokinetics, protein binding and *in vitro* metabolic profile for the first time. Understanding the pharmacokinetic and metabolic aspects of any therapeutic agent is important since its *in vitro* and *in vivo* efficacy is correlated to the systemic concentration and the exposure time. *In vivo* pharmacokinetic studies give an insight into the dose-exposure relationship while the *in vitro* metabolic profile provides useful information regarding elimination mechanism of the compound and rate of biotransformation, both of which decide the residence time of the compound in biological system. Since, it is only the free fraction of the drug that is available for exerting the pharmacodynamics response and that gets distributed and excreted from the body, the plasma protein binding studies becomes important. Thus, the data presented here provide important information to support the *in vivo* efficacy of alda-1 and would be helpful in its further development as a therapeutic agent and synthesis of its analogs with better systemic exposure and disposition properties.

Results and discussion

Optimization of LC-MS/MS conditions:

Full quadrupole scans were carried out in positive ion mode to optimize ESI-MS/MS conditions. During the initial Q1 scan, the protonated molecular ions $[M+H]^+$, $[M+H+2]^+$ and $[M+H+4]^+$ (*m/z* 323.9, 325.9 and 328.0) for Alda-1 were observed in the ratio of 9:6:1 approximately, which is the typical mass spectra peak pattern for chlorine atom. Its sodium adduct was also observed in the similar isotopic pattern in the ratio of 9:6:1 for $[M+Na]^+$, $[M+Na+2]^+$ and $[M+Na+4]^+$. For IS, the protonated molecular ion $[M+H]^+$ appeared at *m/z* 271.6. During the MRM scan, *m/z* 325.9 was found to have better signal-to-noise ratio than 323.9 or 328.0 and was chosen as the precursor ion for

4

quantifying alda-1. Following detailed optimization of mass spectrometry conditions, the transitions m/z 325.9 precursor ion $[M+H]^+$ to the m/z 135.1 product ion and m/z 271.6 precursor ion $[M+H]^+$ to the 137.1 m/z product ion were selected as the quantifier transition for alda-1 and IS, respectively. The transitions m/z 325.9 precursor ion $[M+H]^+$ to the m/z 77.2 product ion and m/z 325.9 precursor ion $[M+H]^+$ to the m/z 51 were selected as the qualifier transitions for Alda-1. The ratio of the qualifier peak to the quantifier peak for m/z 77.2 product ion was found to be 0.175 and 0.170 for m/z product ion 55 and this ratio deviated within ± 5.6% and ± 5.5% for the respective transitions. The Q1 and product ion spectra of alda-1 and IS are shown in figure 2. The fragmentation pattern of alda-1 is shown in figure 3.
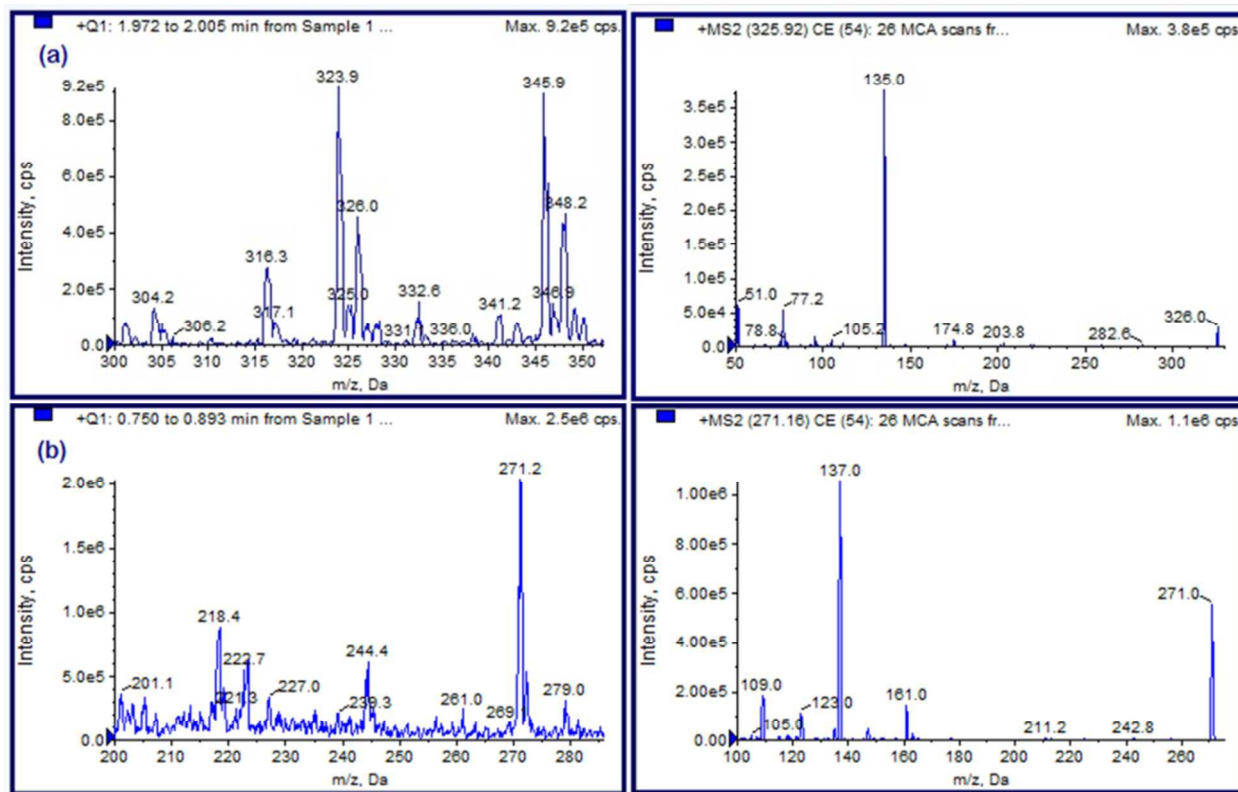

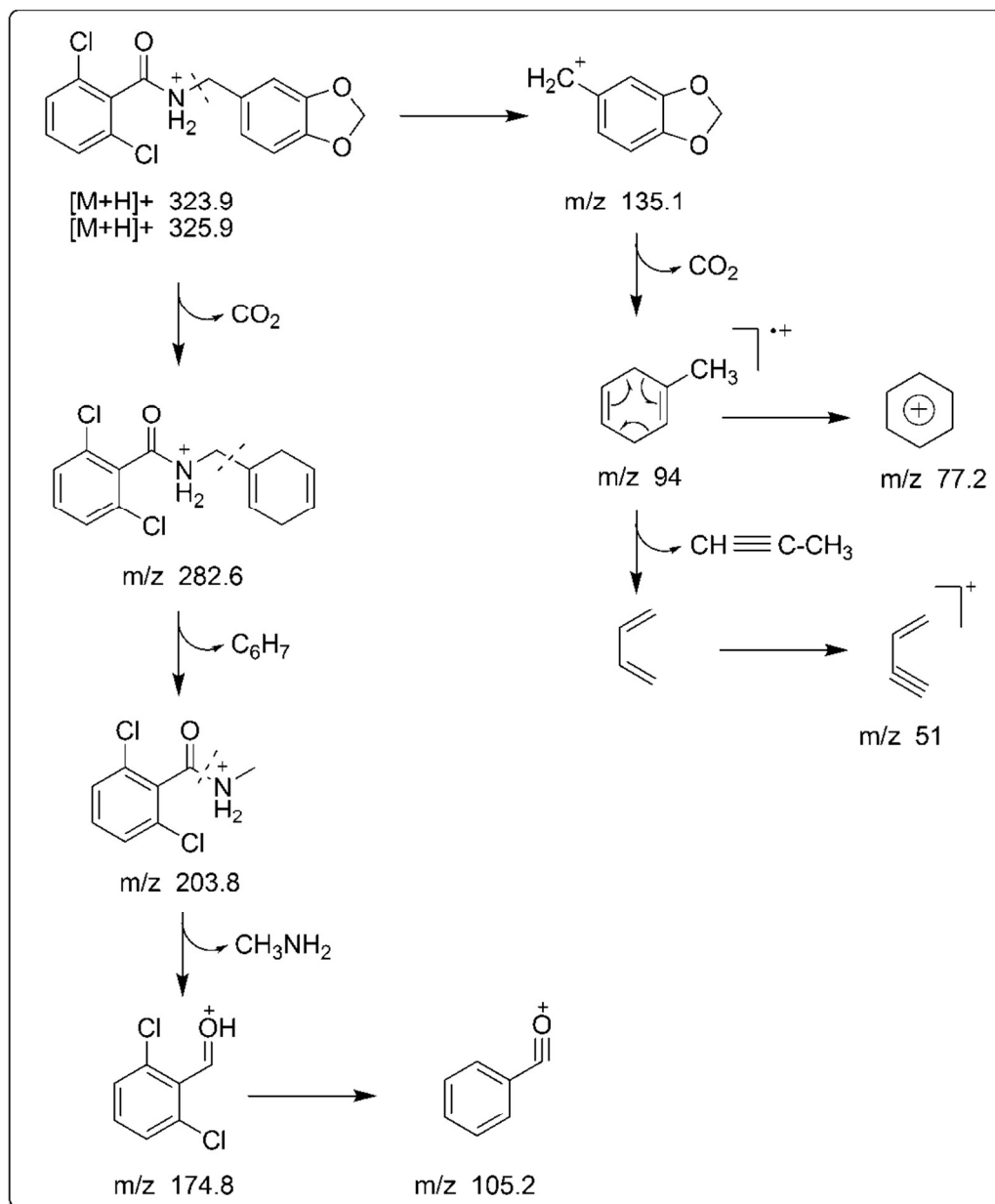**Fig 2** Q1 and product ion scans of (a) alda-1 and (b) IS.

Fig 3 Proposed fragmentation pattern of alda-1.

The LC method was developed by examining the feasibility of various combinations of solvents such as acetonitrile and methanol as organic modifiers; and ammonium acetate, ammonium formate, formic acid and acetic acid as aqueous phase. Different C18 columns were tried along with altered mobile phase compositions and flow rates to achieve good peak shape, sensitivity and selectivity for alda-1 and IS. The LC method was optimized with mobile

phase comprising of methanol and 0.1% formic acid in ratio of 60:40 (v/v) which was delivered at a flow rate of 0.7

mL/min for chromatographic separation on a Waters XBridge® C18 column (4.6 mm × 50 mm, 5.0 µm).

Method validation:

Specificity, matrix effect and carry over:

No endogenous interference or metabolite was found at the LC peak regions of the analytes. Figure 4 shows the

typical chromatograms for blank plasma, plasma spiked with analytes and *in vivo* plasma study sample after 5 h of

intravenous alda-1 administration. The retention time of alda-1 and IS was 2.23 and 3.32 min, respectively. The

matrix effect at the four tested QC concentrations was found to be within 10 % which indicated negligible ion

suppression or enhancement for alda-1. The mean matrix effect for IS at 50 ng/mL was 7.13 %. The carry over

response in the blank sample following the highest calibrator sample was less than 5 % of LLOQ response

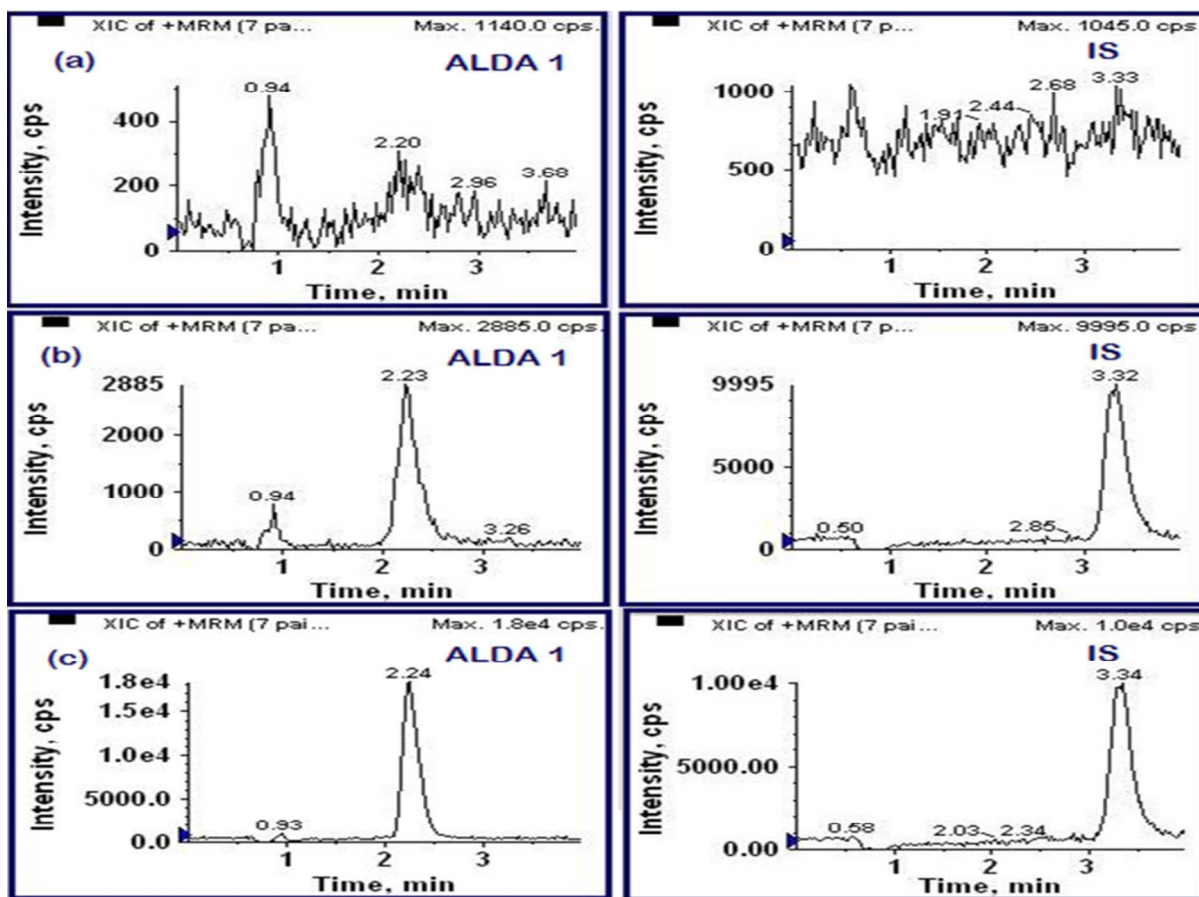suggesting negligible carry over effect.

Fig 4 Typical MRM chromatograms of (a) drug free blank rat plasma, (b) drug free rat plasma spiked with alda-1 at LLOQ (1 ng/mL) and IS and (c) 5 h *in vivo* study sample obtained upon intravenous administration.

Recovery, sensitivity and linearity:

The mean recovery values at the four tested QC concentrations of alda-1 ranged between 91-93 % with acetonitrile, 85-90 % with acidified acetonitrile, 62-71 % with methanol and 68-74 % with acidified methanol while for IS at 50 ng/mL, mean recovery was found to be 97.30 % with acetonitrile. Acetonitrile was, hence, selected as the preferred extraction solvent. The LOD and LLOQ of the method were found to be 0.25 ng/mL and 1 ng/mL, respectively. The calibration curve was found to be linear over the concentration range of 1 to 500 ng/mL (figure 5). The calibration standards were analysed on three separate occasions and the average regression was greater than 0.99 on all occasions. The accuracy of the mean of back calculated concentrations deviated within ± 8 % and the precision was within 9 %.
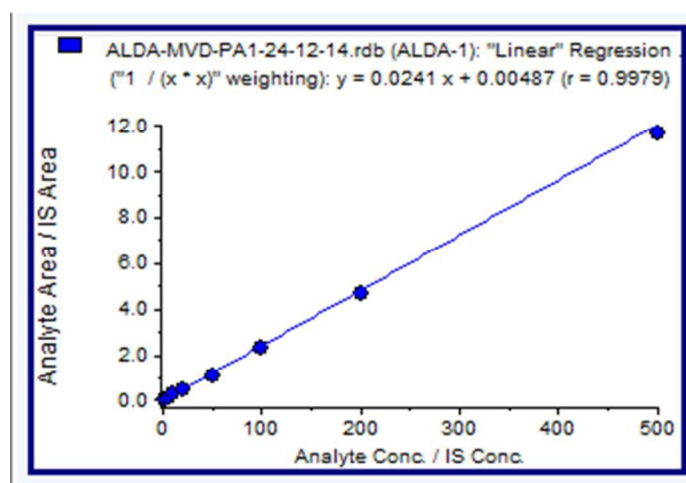

Fig 5 Standard curve for alda-1 within the linearity range of 1-500 ng/mL.

Accuracy and precision:

The intra-day and inter-day accuracy and precision were within the acceptable criteria of ±15%. The results are given in table 1.

Table 1
Intra-day and inter-day assay precision and accuracy for alda-1 in rat plasma (n=6)

	Alda-1 (ng/ml)			
	1 (LLOQ)	**3 (LQC)**	**150 (MQC)**	**420 (HQC)**
	Intra-day			
Day 1				
Mean ± SD	0.91 ± 0.05	2.77 ± 0.11	156 ± 2.97	415.83 ± 20.36
Accuracy (%)[a]	91.12	92.44	104.00	99.01
Precision (%)[b]	5.14	4.06	1.90	4.90
Day 2				
Mean ± SD	1.06 ± 0.04	2.83 ± 0.25	142.83 ± 6.05	398.33 ± 9.44
Accuracy (%)[a]	106.33	94.33	95.22	94.84
Precision (%)[b]	3.29	8.67	4.23	2.37
Day 3				
Mean ± SD	1.01 ± 0.12	3.07 ± 0.36	140.17 ± 6.88	415.83 ± 20.36
Accuracy (%)[a]	100.83	102.33	93.44	99.01
Precision (%)[b]	11.78	11.63	4.91	4.90
	Inter-day			
Mean ± SD	0.95 ± 0.08	2.92 ± 0.29	152.67 ± 7.51	401.61 ± 22.60
Accuracy (%)[a]	94.85	97.41	101.78	95.62
Precision (%)[b]	8.00	9.82	4.92	5.63

[a] Calculated as (mean determined concentration/nominal concentration)×100

[b] Expressed as % R.S.D. (S.D./mean)×100

Stability:

Alda-1 was found to be stable in plasma under all the conditions that could be encountered during sample processing till bioanalysis. The mean variation were within the acceptable limit of 15% for all the QC concentrations. The results are summarized in table 2.

Table 2
Stability of alda-1 in rat plasma (n = 6)

Alda-1 (ng/ml)

	1 (LLOQ)	3 (LQC)	150 (MQC)	420 (HQC)
Bench top stability (6 h)				
Mean ± SD	1.00 ± 0.10	2.97 ± 0.19	150.33 ± 3.20	394.33 ± 19.99
Accuracy	99.16	96.85	96.37	94.83
Precision	10.27	6.52	2.13	5.07
Auto sampler stability (24 h)				
Mean ± SD	0.99 ± 0.13	2.86 ± 0.18	155.50 ± 5.09	444.67 ± 22.92
Accuracy	98.20	93.00	99.68	106.93
Precision	13.51	6.17	3.27	5.16
Long term stability (30 days)				
Mean ± SD	0.92 ± 0.06	2.92 ± 0.30	157.33 ± 6.57	381.17 ± 12.59
Accuracy	91.45	95.22	100.85	91.66
Precision	6.35	10.10	4.18	3.30
Freeze thaw stability (3 cycles)				
Mean ± SD	1.02 ± 0.07	3.14 ± 0.25	138.50 ±7.26	371.50 ± 8.69
Accuracy	101.47	102.28	88.78	89.34
Precision	7.07	8.06	5.24	2.34

In vitro plasma protein binding*:*

The plasma protein binding studies were performed at two concentrations by filtration through anisotropic, hydrophilic YMT ultrafiltration membranes. One of the major drawbacks limiting the use of ultrafiltration technique to protein binding studies is the adsorption of the analyte on to filtration membrane. The non-specific binding studies were thus, carried out. The binding of alda-1 to YMT membrane was found to be insignificant (<10%), thus, validating the use of this technique for alda-1. The correction for non-specific binding was, however, taken into consideration. The mean plasma protein binding of Alda-1 was found to be concentration independent ($p > 0.05$) and ranged between 82-86 % at 100 and 1000 ng/mL.

In vitro metabolic stability and *in vitro-in vivo* extrapolation:

The *in vitro* metabolic stability was performed to understand the biotransformation of alda-1 via cytochrome P450 (CYP450) pathway. Substrate depletion approach was used to estimate the *in vitro* $t_{1/2}$, since the metabolites of alda-1 were unknown at this stage. The results are summarized in table 3. The mean hepatic clearance in rats was found to be 53.49 mL/min/kg (1.28 L/h/kg) and the mean hepatic extraction ratio was 0.76. The degradation profile of

alda-1 in rat liver microsomes is given in figure 6. Around 10 % of the parent remained in the microsomal reaction mixture after 60 minutes incubation.
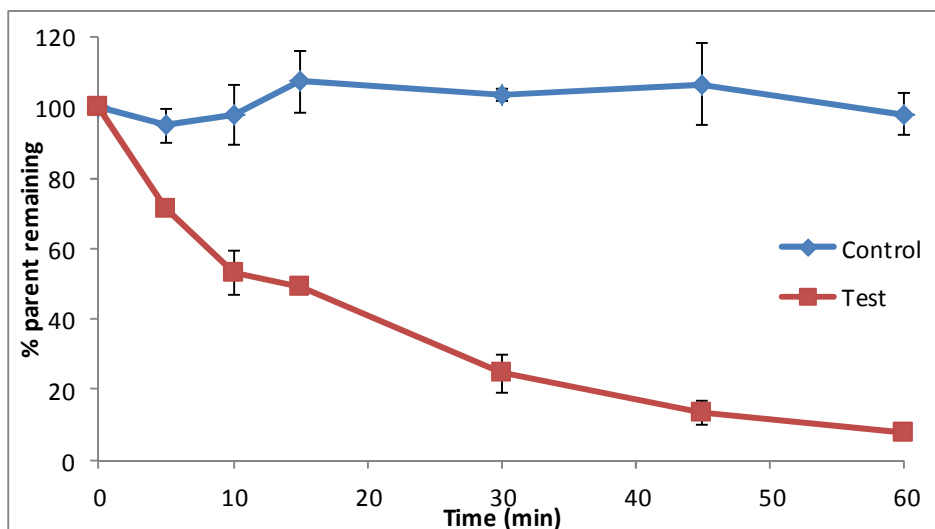

Fig 6 Degradation profile of alda-1 upon incubation with rat liver microsomes (mean ± S.D).

Table 3
In vitro pharmacokinetic parameters of alda-1

Parameter	Alda-1
Plasma protein binding (%)	82.83 ± 1.81 (at 100 ng/mL)
	85.94 ± 2.15 (at 1000 ng/mL)
***In vitro* $t_{1/2}$ (min)**	11.01 ± 0.93
Intrinsic clearance (CL_{int}) (μL/min/kg)	126.3 ± 10.63
Whole liver clearance (mL/min/kg)	227.34 ± 19.14
Hepatic clearance (mL/min/kg)	53.49 ± 1.06
Hepatic extraction ratio	0.76 ± 0.01

In vivo pharmacokinetics*:*

The developed method was used to determine the pharmacokinetics of alda-1 in female SD rats after oral and intravenous administration. The data was subjected to non-compartmental analysis. The mean plasma concentration-time profiles are presented in figure 7 and the pharmacokinetic parameters in table 4.
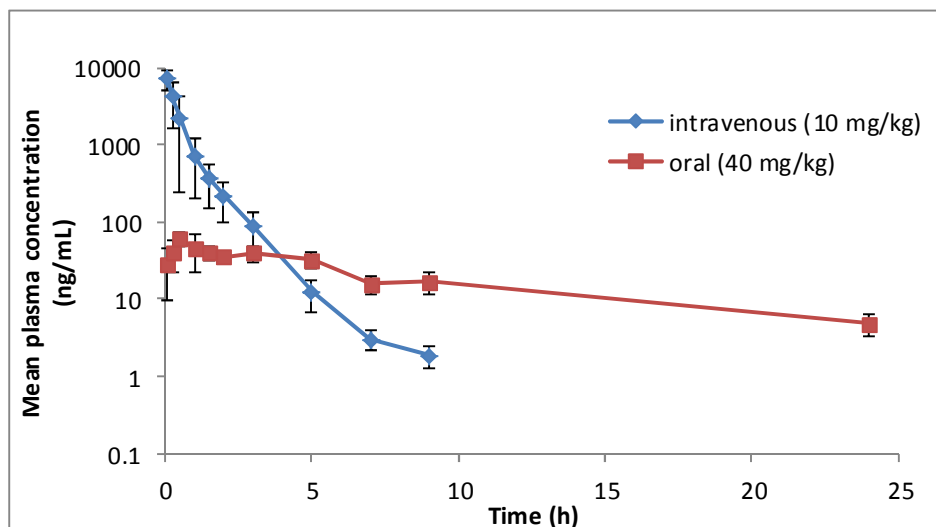
Fig 7 Mean plasma concentration-time plots alda-1 following intravenous (10 mg/kg) and oral (40 mg/kg) administration (mean ± S.D).

Table 4
Pharmacokinetic parameters of alda-1 after intravenous and oral administration in female SD rats (n = 4).

Parameter	Intravenous (10 mg/kg)	Oral (40 mg/kg)
$t_{1/2}$ (h)	1.67 ± 0.54	7.33 ± 1.37
C_o (ng/mL)	8774.71 ± 955.72	--
C_{max} (ng/mL)	--	69.88 ± 4.60
T_{max} (h)	--	0.63 ± 0.25
V_d (L/kg)	9.39 ± 1.80	41.50 ± 2.95
CL (L/h/kg)	3.97 ± 0.54	4.01 ± 0.72
AUC_{0-t} (h*ng/mL)	2536.07 ± 347.25	415.83 ± 57.04
$AUC_{0-\infty}$ (h*ng/mL)	2540.80 ± 246.19	469.12 ± 81.24
Bioavailability (%)	--	4.62

Upon oral administration, the peak concentration was reached at 0.63 h and was found to be 69.88 ng/mL. This suggests that alda-1 has a high absorption rate. It was found to have a low oral bioavailability of 4.62 %. Upon intravenous administration, alda-1 was detectable up to 9 h only. The high clearance, 3.97 ± 0.54 L/h/kg, of alda-1 suggest it to be a high extraction compound. However, the *in vivo* clearance was found to be significantly higher

than the hepatic clearance (1.28 L/h/kg) obtained from microsomal studies. This implies that apart from CYP-mediated phase I biotransformation, other elimination mechanisms such as phase II biotransformation or renal elimination might also be involved that are responsible for the high *in vivo* clearance of this compound. The high hepatic extraction of alda-1 and its poor bioavailability could prove to be a limiting factor in clinical development of this compound. During the reanalysis of the incurred samples, all the samples were found to be within ± 15 % of the mean value, thus, re-establishing the validity and robustness of the developed method.

Materials and methods

Chemicals and reagents:

Alda-1 was provided by Dr. Naibedya Chattopadhyay from Division of Endocrinology, CSIR-Central Drug Research Institute (Lucknow, India). Medicarpin (internal standard, IS) was synthesized at the Medicinal Chemistry Division of Central Drug Research Institute (Lucknow, India). Chemical structures of alda-1 and IS are shown in Figure 1. HPLC grade methanol, acetonitrile and sodium carboxy methyl cellulose (CMC) were purchased from Sigma Aldrich Ltd (St Louis, USA). Formic acid AR was purchased from E Merck Limited (Mumbai, India). Centrifree® micro partition system (product No. 4104) was purchased from Amicon Inc. (MA, USA). Ultra pure water was obtained from a Sartorious Arium 611 system. Heparin sodium injection I.P. (1000 IU/mL, Biologicals E. Limited, Hyderabad, India) was purchased from local pharmacy.

Animals and prerequisites: Blank, drug free plasma samples were collected from adult, healthy female *Sprague–Dawley* (SD) rats at the Division of Laboratory Animals (DOLA) of Central Drug Research Institute (Lucknow, India). Prior approval from the Institutional Animal Ethics Committee (IAEC) was sought for maintenance, experimental studies, euthanasia and disposal of carcass of animals. Plasma was obtained by centrifuging the heparinised blood (25 IU/mL) at 2000×g for 10min at 20°C.
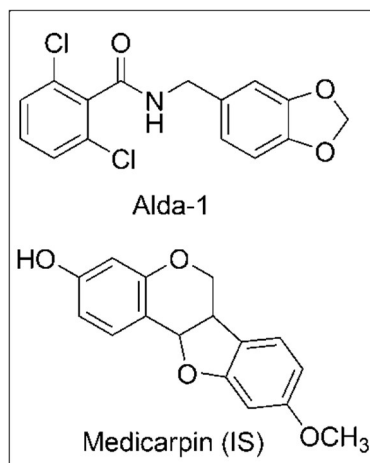
Fig 1 Chemical structures of alda-1 and medicarpin (IS).

Instrumentation and LC-ESI-MS/MS conditions:

Chromatographic separation was performed on a Waters XBridge® C18 column (4.6 × 50 mm, 5.0 μm) using Shimadzu UFLC consisting of SCL 20Avp pump and SIL-HTc autosampler. The system was run in isocratic mode with mobile phase consisting of methanol : 0.1% formic acid in the ratio of 60:40 (v/v) at a flow rate of 0.7 mL/min. Mobile phase was duly filtered through 0.22 μm Millipore filter (Billerica, USA) and degassed ultrasonically for 15 min prior to use. Separations were performed at room temperature. The injection volume was kept at 20 uL.

Mass spectrometric detection was performed in multiple reaction monitoring (MRM) mode on an API 4000 QTRAP mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source and triple quadrupole mass analyser. Quadrupoles Q1 and Q3 were set on unit resolution in positive ion mode. The ion spray voltage was set at 5500 V. The instrument parameters viz., nebulizer gas, curtain gas, auxillary gas and collision gas were set at 50, 20, 50 and 8 psi, respectively. Compound related parameters viz., declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 66, 29, 10, 26 V; and 50, 25, 10, 10 V for Alda-1 and IS, respectively. Zero air was used as source gas while nitrogen was used as both curtain and collion gas. The transition of m/z 325.9 precursor ion $[M+H]^+$ to the m/z 135.1 product ion was monitored for Alda-1 and m/z 271.6 precursor ion $[M+H]^+$ to the 137.1 m/z product ion for IS. Data acquisition and quantitation were performed using Analyst® software version 1.6 (Applied Biosystems, MDS Sciex Toronto, Canada).

Stocks and working standards:

A primary stock solutions of alda-1 and IS (1mg/mL) were prepared in methanol. Working standard solutions were prepared by combining the aliquots of each primary stock solution and diluting with methanol. All the solutions were stored at -20°C. The stability of the stock solutions were confirmed by comparing them with the freshly prepared stock solutions.

Calibration standards and quality control samples:

Calibration standards of Alda-1 were prepared by spiking 5 µL of appropriate working stocks in 95 µL pooled blank rat plasma to obtain nine calibration standards from 1 – 500 ng/mL. Calibration curves were obtained by plotting the peak area ratios of the analyte to IS versus the analyte concentration. The curves were fitted using linear regression with $1/x^2$ weighting. The quality control (QC) samples were prepared at four concentrations levels, 1 ng/mL (lower limit of quantitation, LLOQ), 3 ng/mL (low QC), 150 ng/mL (medium QC) and 420 ng/mL (high QC), by following the same procedure as for calibration standards. The lowest limit of detection (LOD) was defined as the lowest concentration with signal-to-noise ratio more than 3. The LLOQ was defined as the lowest concentration with signal-to-noise ratio more than 10 and having accuracy and precision within ±20%. All samples were stored at -70°C until analysis.

Sample preparation:

A simple protein precipitation technique was employed for extracting Alda-1 from rat plasma. To 100 µL of rat plasma, 200 µL of acetonitrile (containing IS, equivalent to 50 ng/mL) was added. The mixture was vortexed for 5 minutes followed by centrifugation for 10 min at 13,000 g on Sigma 3-16K (Frankfurt, Germany). The supernatant was separated and 20 µL was injected for LC-MS/MS analysis.

Validation:

The assay was validated following the bioanalytical method validation guidelines issued by the FDA Center for Drug Evaluation and Research (CDER) [7]. The parameters recovery, specificity, accuracy, precision, matrix effects, dilutional integrity and stability were evaluated using drug-free plasma from six different rats at four QC levels (LLOQ, QC low, QC medium and QC high). Incurred sample reanalysis was also performed to increase the reliability of the developed method.

15

Specificity was tested by analyzing plasma samples spiked with Alda-1 and IS in comparison to six randomly selected blank plasma samples to investigate potential interferences at the LC peak region of both the analytes. Recovery was assessed by comparing the peak responses of the pre-extracted plasma samples to those of post-extracted blank plasma samples spiked with equivalent concentration of the analytes. The recovery of IS was determined at 50 ng/mL. The matrix effect was evaluated by comparing the peak response of the post extracted spiked plasma samples and the unextracted analytical neat samples at equivalent concentrations [8-9]. The intraday accuracy and precision were determined by six repeat analyses (intrabatch variability) of the four QC concentrations. The interday accuracy and precision were determined by analyzing four QC concentrations in three different runs (interbatch variability) on 3 days. Accuracy should lie within $\pm$ 15% deviation of the nominal value and precision within $\pm$ 15% relative standard deviation (RSD) within except for LLOQ where both should lie within $\pm$ 20%. The stability of plasma samples was assessed under different storage conditions: bench top (BT) stability at room temperature for 6 h; long term (LT) stability at -80°C for 30 days, autosampler (AS) stability at 4°C for 24 h and; freeze/thaw (FT) stability after 3 freeze-thaw cycles. Autosampler carry over was determined in triplicate by injecting the highest calibration standard followed by a blank sample and was considered insignificant if the measured peak area was less than 20% of the lowest calibrator area. Dilution integrity was performed by 20 times dilution of the plasma samples containing 8000 ng/mL of Alda-1 with blank plasma to obtain plasma samples containing 400 ng/mL of Alda-1. The response after dilution should not vary more than 15% of the true value. An incurred sample reanalysis (ISR) was performed by selecting the *in vivo* pharmacokinetic study samples, to show reproducibility of method. Four samples were selected from each rat including the time points at Cmax and elimination phase to cover the entire profile of the individual subject. Thus, the ISR was performed on 24 samples out of total 88 samples. The difference between the original and reanalyzed concentrations of the analyte should be within 20% for at least 67% of the total samples reanalyzed [7].

$$\% \text{ Difference } = \frac{\text{Reanalyzed concentration} - \text{ Original concentration}}{\text{Mean of original and reanalyzed concentration}} \text{ X } 100$$

In vitro plasma protein binding:

The plasma protein binding of alda-1 was estimated in triplicate by ultra-filtration method using centrifree® micro partition system (Amicon Inc., MA, USA). Freshly collected rat plasma was spiked with alda-1 (stock solutions

were prepared as described above) to obtain the plasma concentrations of 100 and 1000 ng/mL. Spiked plasma

allowed to equilibrate for 15 min before the start of the study. Samples in triplicate were placed in centrifree® tubes

and centrifuged at 3500 x g for 15 min at 37 ºC. 100 ul each of initial plasma samples and ultra-filtrates were

subjected to LC-MS/MS after sample treatment as described previously [10]. The non-specific binding (NBS) to the

filtration membrane was estimated by spiked equivalent concentration in phosphate buffer saline (PBS) and further

processing the samples similar to the plasma samples. The plasma protein binding was determined by using the

formula:

$$\% \text{ Protein binding} = 1 - \left(\frac{Cf}{Co}\right)(1 + NBS) \times 100$$

where C_O is the dosing concentration of the compound; Cf is the concentration in the filtered matrix and; NBS is the

non-specific binding calculated as 1- ($C_{p,f}$ / $C_{p,uf}$) where $C_{p,f}$ is the average concentration in the filtered PBS and $C_{p,uf}$

is the average concentration in the unfiltered PBS. Student's t-test of significance was applied to compare the effect

of concentration on plasma protein binding of alda-1.

In vitro metabolic stability:

Metabolic stability of alda-1 was performed in triplicate using glass tubes [11]. To each tube, 460 µL of phosphate

buffer (0.1 M, pH 7.4) and 12.5 µL of rat liver microsomal protein (20 mg/mL) were added and incubated for at

37±0.2°C for 5 min. Then, 2.5 µL of test compound (1mM) was added. For positive control testosterone was used as

the test compound. In negative control, NADPH was replaced by 25 µL of phosphate buffer. Reaction was initiated

by addition of 25µL of NADPH (24 mM) and terminated at 0, 5, 10, 15, 30, 45 and 60 min by addition of 450 µL

ice-cold acetonitrile to 50 µL reaction mixture, followed by centrifugation for 15 min at 10,000 rpm. 20µL of

supernatant was directly analyzed by LC-MS/MS. The data was analysed using GraphPad Prism 5.0 software. Non-

linear regression of percent parent remaining against time was performed as per the equation:

$$\% \text{ Parent drug remaining} = \text{Span}*e^{-kt} + \text{Plateau}$$

where 'Span' denotes the differences in the % parent drug remaining at time zero to time t, 'Plateau' is the % parent

drug remaining at time tlast and 'k' is the rate constant for metabolism. The *in vitro* half-life (*in vitro* $t_{1/2}$) was

determined as 0.693/k.

The *in vitro* parameters were estimated using the following formulae:

$$Intrinsic\ clearance\ (Clint) = k\ \times \frac{Volume\ of\ incubation\ mixture}{Amount\ of\ protein\ incubated}$$

$$Whole\ liver\ clearance\ (CLint) = k\ \times \frac{Liver\ weight}{Body\ weight} \times SF$$

$$Hepatic\ clearance\ (CLh) = \frac{Q\ \times CLint}{Q + CLint}$$

$$Hepatic\ extraction\ ratio = \frac{CLh}{Q}$$

Where SF is the scaling factor defining microsomal protein per gram liver and was taken as 45 for rats. Q is the blood flow in rats taken as 70 mL/min/kg.

In vivo pharmacokinetic study:

Female SD rats were divided into two groups (n=4 each): one group (for intravenous pharmacokinetic study) was provided free access to food and water; while the other group was fasted overnight (12-14 h) prior to the oral pharmacokinetic study and had free access to water throughout the experimental period. Alda-1 was administered orally at a dose of 40 mg/kg in 0.25% CMC suspension and intravenously as a solution (prepared in 40% dimethyl formamide, 30% polyethylene glycol, 20% triple distilled water and 10% ethanol) at a dose of 10 mg/kg. Animals were provided with standard diet 3 h after oral dosing. The rats were partially anaesthetized using diethyl ether and blood samples were collected from the retro-orbital plexus into heparinized microfuge tubes at 0.083, 0.25, 0.5, 1, 1.5, 2, 3, 5, 7, 9 and 24 h post-dosing. Plasma was harvested by centrifuging the blood at 13000 rpm for 10 min on Sigma 1-15K (Frankfurt, Germany). Samples were stored at $-80 \pm 10°C$ until bioanalysis. All the four QC concentrations (n=5 each) were analysed with each batch of study samples. The observed maximum serum concentration (C_{max}) and the time to reach the maximum serum concentration (T_{max}) were obtained by visual inspection of the experimental data. The data was subjected to non-compartmental pharmacokinetics analysis using WinNonlin (version 5.1, Pharsight Corporation, Mountain View, USA). Area under curve (AUC) from 0 to 24 hr

(AUC_{0-24}) was calculated using linear trapezoidal rule. AUC from 0 to infinity $(AUC_{0-\infty})$ was calculated as the sum of AUC_{0-t} and C_{last}/k_{el}, where, C_{last} represents the last quantifiable concentration and k_{el} represents the elimination rate constant.

Conflict of interest

The authors declare that they have no conflict of interest.

Conclusion

In the present study, an accurate, sensitive and robust LC-ESI-MS/MS method was developed and validated to quantify alda-1 in plasma biomatrix for the first time. The sensitivity of the method was 1 ng/mL. The method was successfully applied to study the oral and intravenous pharmacokinetics alda-1. Alda-1 was found to be poorly bioavailable. The same method was also used to study its *in vitro* CYP stability and plasma protein binding. Alda-1 was found to be a high extraction compound and was 82-85% plasma protein bound. The study provides groundwork to carry forward the research in this direction.

References

[1]   Chen C H, Budas G R, Churchill E N, Disatnik M H, Hurley T D, Mochly-Rosen D (2008) Science 321:1493-1495.

[2]   Budas G R, Disatnik M H, Mochly-Rosen D (2009) Trends Cardiovas Med 19:158-164.

[3]   Perez-Miller S, Younus H, Vanam R, Chen C H, Mochly-Rosen D, Hurley T D (2010) Nat Struct Mol Biol 17:159-164.

19

[4]   Beretta M, Gorren A C, Wenzl M V, Weis R, Russwurm M, Koesling D, Schmidt K, Mayer B (2010) J Bio

        Chem 285:943-952.

[5]   Chen C H, Sun L, Mochly-Rosen D (2010) Cardiovas Res 88:51-57.

[6]   Sun A, Zou Y, Wang P, Xu D, Gong H, Wang S, Qin Y, Zhang P, Chen Y, Harada M, Isse T, Kawamoto

        T, Fan H, Yang P, Akazawa H, Nagai T, Takano H, Ping P, Komuro I, Ge J (2014) J Am Heart Assoc

        3:e000779.

[7]   Guidance for Industy : Bioanalytical Method Validation, US Food and Drug Administration, **2013**.

[8]   Singh S P, Wahajuddin, Ali M M, Kohli K, Jain G K (2011) J Chrom B 879:2845-2851.

[9]   Wahajuddin, Singh S P, Jain G K (2009) J Chrom B 877:1133-1139.

[10]   Singh S P, Wahajuddin, Tewari D, Pradhan T, Jain G K (2011) Food Chem Tox 49:1056-1062.

[11]   Baranczewski P, Stanczak A, Sundberg K, Svensson R, Wallin A, Jansson J, Garberg P, Postlind H (2006)

        Pharmacol Rep : PR 58:453-472.