ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
22 (2000) 461–467

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Determination of linsidomine in human plasma by tandem LC–MS with ESI

F.C.W. Sutherland *, A.D. de Jager, K.J. Swart, H.K.L. Hundt, T. Scanes, A.F. Hundt

Department of Pharmacology, G6, FARMOVS Research Centre for Clinical Pharmacology and Drug Development, University of the Orange Free State, P.O. Box 339, Bloemfontein 9300, South Africa

Received 10 May 1999; received in revised form 16 September 1999; accepted 20 September 1999

Abstract

A sensitive method for the determination of linsidomine in plasma was developed, using high-performance liquid chromatographic (HPLC) separation with tandem mass spectrometric detection. Linsidomine was derivatised with propyl chloroformate and extracted with *tert*-butyl methyl ether/1,2-dichloroethane (55:45, v/v), back-extracted into HCl (0.01 M) followed by alkalinisation and back-extraction into ether; the final ether extract evaporated, reconstituted in mobile phase and then separated on a Phenomenex® Luna C18 (2) 5 μ 2.1 × 150 mm column with a mobile phase consisting of methanol–water–formic acid (98/100%) (400:600:0.05, v/v/v) at a flow-rate of 0.4 ml min^{-1}. Detection was achieved by a Finnigan MAT mass spectrometer (LCQ) at unit resolution in the selected reaction monitoring (SRM) mode monitoring the transition of the protonated molecular ion m/z 257.0 to the product ion m/z 86.0. The mean recovery for linsidomine was 51% with a lower limit of quantification of 0.70 ng/ml using 1 ml plasma for extraction. This LC–MS/MS method for the determination of linsidomine in human plasma allows for better specificity and a higher sample throughput than the traditional LC–UV methods. It also demonstrates the profound effect that the composition of acidic modifiers and matrix constituents can have on the electrospray ionisation (ESI) of the analyte.

Keywords: Linsidomine; Plasma; Electrospray; Liquid chromatography; Mass spectrometry

1. Introduction

Linsidomine, 3-morpholinosydnonimine, is an active metabolite of the vasodilating agent, molsidomine (*N*-ethoxycarbonyl-3-morpholinosydnonimine). The usual dose is 4 mg orally with resultant maximum plasma concentrations of about 20 ng ml^{-1}.

Linsidomine is rapidly transformed into *N*-nitroso-*N*-morpholinoaminoacetonitrile (SIN-1A) which can yield a pharmacologically active NO^- radical resulting in SIN-1A being non-enzymati-

* Corresponding author. Fax: +27-51-4471779.
E-mail address: gnfmfcws@frm.uovs.ac.za (F.C.W. Sutherland)

0731-7085/00/$ - see front matter
PII: S0731-7085(00)00239-9

cally transformed to *N*-cyanomethylaminomorpholine (SIN-1C) [1].

Dell and Chamberlain [2] developed the first chromatographic method for the determination of molisidomine in plasma using high-performance liquid chromatography (HPLC) with UV detection, as the polarity of the mesionic ring system of molsidomine made gas chromatography unlikely. The method was however not sensitive enough and could not be used to determine the active metabolite (linsidomine) of molsidomine. We based our initial analytical method on a method described by Dutot et al. [3] using UV detection. They used a similar UV detection method proposed by Koyama et al. [4] for the simultaneous determination of molsidomine and linsidomine in one chromatographic run. They combined previously described extraction methods [5,6] which involves the formation of alkoxycarbonyl linsidomine derivatives to allow separation on reversed-phase HPLC and extraction in a slightly polar organic solvent. Although, the method worked well for molsidomine we experienced problems with specificity and sensitivity for linsidomine. The retention time for the linsidomine derivative was 8.5 min compared to 4.5 min for molsidomine. The longer retention times for the linsidomine derivative resulted in broader peaks, which resulted in higher limits of quantification, which were unacceptable for pharmacokinetic studies. There was also a constant interfering peak that eluted at the same retention time as the linsidomine derivative, which caused problems in quantifying linsidomine at the lower concentrations. To enhance specificity and sensitivity, we developed a method on a mass-selective detector with mass spectrum/mass spectrum (MS/MS) capabilities in tandem with liquid chromatography (LC) allowing the determination of linsidomine in plasma in a 4.5 min chromatographic run. In order to obtain a suitable solvent for injection onto the LC–MS, the extraction method had to be adapted. For this study, we only determined linsidomine but the method could easily be used for the rapid and simultaneous determination of molsidomine and linsidomine in one chromatographic run.

2. Experimental

2.1. Materials and chemicals

2.1.1. Column

A Phenomenex® Luna C18 5 μ, 2.1 × 150 mm column (Phenomenex, Torrance, CA) was used for separation at a flow-rate of 0.4 ml min^{-1} and injecting 50 μl onto the column. The mobile phase was delivered by a Hewlett-Packard Series 1100 pump and the samples injected by a Hewlett-Packard Series 1050 autosampler (Hewlett-Packard, Palo Alto, CA). Detection was performed by a Finnigan Mat LCQ™ MS^n detector (Finnigan Mat, San Jose, CA) using electrospray ionisation (ESI) for ion production.

Hydrochloric acid, dipotassium hydrogen phosphate and 1,2-dichloroethane (Pro-Analysi) were obtained from Merck (Darmstadt, Germany); diethylether and methanol (Burdick and Jackson, High Purity) were obtained from Baxter chemicals, citric acid (AnalaR) and formic acid (98/100%) were obtained from BDH Laboratory Supplies (Poole, UK), *tert*-butyl methyl ether (99.7%), propyl chloroformate (98%) and butyl chloroformate (98%) were obtained from Aldrich Chemical Company Inc., Milwaukee, WI, and crystallised tris (hydroxymethyl)-aminomethane was obtained from Boehringer Mannheim GmbH, Germany. All chemicals were used as received. Water was purified by RO 20SA reverse osmosis and Milli-Q® polishing system (Millipore, Bedford, MA).

A tris (hydroxymethyl)-aminomethane buffer (0.5 M) was prepared and adjusted to pH 8.5 with concentrated hydrochloric acid. A citrate buffer was prepared by adjusting 1.5 M citric acid to pH 2.1 with 1 M dipotassium hydrogen phosphate.

Linsidomine, $C_6H_{10}N_4O_2$, was supplied by Francochim. Derivatised linsidomine stock solutions were prepared according to the method of Dutot et al. [3].

2.2. Extraction procedure

The following extraction and derivatisation method was adapted for LC–MS determinations from a HPLC method with UV detection used by

Dutot et al. [3]. Linsidomine standard solutions were made up in methanol and used immediately to spike plasma and discarded thereafter. Calibration standards and quality control standards were prepared in normal human plasma containing citrate buffer (pH 2.1; 1.5 M), (2.5 ml buffer to 200 ml plasma), by spiking a pool of normal plasma, which was then serially diluted with normal blank plasma to attain the desired concentrations (0.7–41.09 ng/ml). An equivalent value of citrate buffer was added to the trial samples. The calibration standards and quality control standards were aliquoted into tubes and stored under the same conditions as the trial samples, in the dark at approximately −70°C.

To 1 ml plasma in a 10 ml amber ampoule was added 50 μl acidic methanol (1:1000, v/v), 50 μl derivatising agent (propyl chloroformate), 5 ml *tert*-butyl methyl ether-1,2-dichloroethane (55:45, v/v) and 1 ml tris buffer solution (pH 8.5; 0.5 M). These steps were done in rapid succession without homogenising in between. The sample was then immediately vortex mixed for 2 min and centrifuged at 1300 × *g* for 5 min at 10°C. No internal standard was used in this method.

The aqueous phase was frozen at −30°C on a Fryka Polar cooling plate (Kältetechnik, Esslingen), the organic phase decanted into a clean amber glass ampoule and evaporated under vacuum on a Savant SpeedVac® (Savant Instruments Inc., NY) rotary evaporator at 40°C. Diethyl ether (3 ml) and 200 μl hydrochloric acid (0.01 M) were added to the residue and the solution was vortex mixed for 2 min and centrifuged at 1300 × *g* for 5 min at 10°C. The aqueous phase was frozen at −30°C on a Fryka Polar cooling plate and the organic phase discarded. Tris buffer (100 μl, pH 8.5; 0.5 M) and 3 ml diethyl ether were added to the aqueous phase and the solution was vortex mixed for 2 min and centrifuged at 1300 × *g* for 5 min at 10°C. The aqueous phase was frozen at −30°C on a Fryka Polar cooling plate, the organic phase decanted into a clean amber glass ampoule and evaporated under vacuum on a Savant SpeedVac® rotary evaporator at 40°C.

The residue was dissolved in mobile phase (200 μl), the solution transferred to an amber autosampler vial containing a 250 μl glass insert for injection onto the analytical column. To prevent possible degradation of the analyte, samples were kept cold at approximately 2°C on the autosampler by circulating refrigerated water with a Lauda RM-6 cooling device (Lauda Dr R Wobser GmbH & Co., KG, Lauda-Königshofen). The entire extraction procedure was performed under sodium vapour lamp illumination.

2.3. Liquid chromatography

All chromatographic solvents were sparged with helium before use. Chromatography was carried out at ambient temperature at a flow-rate of 0.4 ml/min with methanol–water–formic acid (400:600:0.05, v/v/v) as mobile phase. The column outlet was connected to a 100 μm I.D. fused-silica capillary, which transferred the whole eluent into the ion source.

2.4. Mass spectrometry

ESI was performed in the positive ion mode with nitrogen as the nebulizing (75 U) and auxiliary gas (8 U). For tuning, a T-piece was installed in the flowline from the HPLC before the connection to the silica capillary, and connected to the syringe pump of the instrument via a piece of PEEK tubing. The response of the instrument for derivatised linsidomine was optimised by injecting a constant flow of a solution of the drug in mobile phase into the stream of mobile phase eluting from the column. The response was optimal with a spray voltage setting of 3.70 kV, which produced a spray current of 0.82 μA for this specific mobile phase. The heated capillary voltage was set at 13.0 V and the temperature to 200°C.

The instrument was operated at unit resolution in the selected reaction monitoring mode (SRM), monitoring the transition of the protonated molecular ion m/z 257.0 to the product ion m/z 86.0. For experiments done with underivatised linsidomine the instrument was operated at unit resolution in the selected reaction monitoring mode (SIM), monitoring the protonated molecular ion m/z 171.7. The maximum inject time was set at 600 ms and the total microscans at 1. The isolation width was 2 amu and the relative colli-

sion energy set at 7%. The LCQ was interfaced to a computer workstation running Finnigan Mat LCQ Navigator software.

2.5. Validation

The method was validated by analysing plasma quality control samples five times at seven different concentrations i.e. 36.4, 20.1, 10.2, 3.37, 2.51, 1.69 and 0.83 ng ml^{-1} to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve containing eight different concentrations spanning the concentration range (0.70–41.09 ng ml^{-1}). Calibration graphs were constructed using a weighted linear regression of the peak-areas of the product ion (m/z 86) for linsidomine versus nominal drug concentrations (weighted 1/concentration).

The matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionisation) was investigated by extracting 'blank' biological fluids from six different sources, reconstituting the final extract in mobile phase containing a known amount of the analyte, analysing the reconstituted extracts and then comparing the peak areas of the analyte.

Absolute recoveries of the analyte were determined in triplicate in normal plasma by extracting drug-free plasma samples spiked with linsidomine. Recoveries were calculated by comparison of the analyte peak areas of the extracted samples with those of the unextracted response standard mixtures representing 100% recovery. The recovery was calculated using the response standard since no difference in ionisation between extracted samples and pure solutions was observed.

On-instrument stability was determined by plotting the quality control standards response factors versus the injection time in the validation batch.

3. Results and discussion

The mean absolute recoveries of analyte determined in triplicate at 36.4, 10.2 and 0.83 ng ml^{-1} were 49.2, 51.6 and 41.7%, respectively. These values are lower than the values reported by Dutot et al. [3] (70–82%) and is probably due to the additional extraction steps included in our method. We had to adapt the method since HCl cannot be used as the injection solvent for LC–MS determinations as with HPLC determinations. We also found that the ionisation of linsidomine was very sensitive to any residual derivatising reagent remaining in the extracts. It was therefore necessary to include another back-extraction in our method, which then gave reproducible results. No matrix effect was observed for the six different plasma pools. The peak areas of the six reconstituted samples had a coefficient of variation of 4.17% indicating that the extracts were 'clean' with no undetected co-eluting compounds that could influence the ionisation of the linsidomine derivative.

The lower limit of quantification (LLOQ) was determined from the data obtained for the assayed quality controls during pre-study validation, since these data included determinations of the analyte at concentrations close to the limit of detection as well as from the results obtained from the quality controls which were processed with each batch of samples run. The LLOQ was finally set at 0.70 ng/ml, i.e. at the value determined during the pre-study validation.

Results from the intra-day validation assays indicate a valid calibration range 0.70–41.09 ng ml^{-1}. Table 1 shows the quality control data obtained during the validation of the method (intra-day), while Table 2 depicts the inter-day back-calculated calibration standards, indicating a valid calibration range from 0.70 to 41.09 ng ml^{-1}.

On-instrument stability was inferred from intra-day quality control data obtained during the pre-study validation. No significant degradation could be detected in the cooled samples left on the autosampler for at least 12 h. According to Dutot et al. [3] molsidomine and linsidomine is stable in buffered plasma (pH 5.4) at -20°C for 12 months.

Due to the high specificity of MS/MS detection, no interfering or late eluting peaks were found when chromatographing the blank plasma extracts from six different sources.

Table 1
Summary of quality control results of linsidomine in human plasma as obtained during the validation (intra-day variation)

Nominal concentration (ng ml^{-1})	Linsidomine ($n = 5$) mean concentration found (ng ml^{-1})	RSD (%)	% NOM
36.4	36.0	5.20	99.0
20.1	20.4	16.0	101.2
10.2	10.7	4.50	104.6
3.37	3.30	6.90	97.9
2.51	2.56	12.2	102.0
1.69	1.74	12.3	103.1
0.83	0.81	15.3	97.6

Table 2
Summary of back-calculated calibration standards concentrations of linsidomine (13 batches) showing the repeatability of the method (inter-day variation)

Nominal (ng ml^{-1})	0.70	1.38	2.01	6.86	13.7	20.0	27.4
Mean	0.68	1.40	1.91	7.42	13.8	19.9	26.8
RSD	13.0	10.8	8.4	8.3	8.7	8.2	6.1
% NOM	97.5	101.7	95.1	108.2	101.2	99.3	97.5

Different concentrations of acetic acid, formic acid and volatile buffers were tested for optimum ionisation. The ionisation of linsidomine proved to be very dependent on the exact amount of acid added to the mobile phase. Addition of only 50 μl formic acid to 1 l of mobile phase gave the best results. Any increase or decrease in the amount of formic acid led to a drastic decrease in the response of linsidomine. Underivatised linsidomine gave a lower response than derivatised linsidomine.

Fig. 1 shows the single parent (m/z 257.0) to product ion MS/MS of the propoxycarbonyl linsidomine derivative acquired with the abundant product ion at m/z 86 which represents the morphilino group of linsidomine. Similar results were obtained when derivatisation was done with butyl chloroformate where the butoxycarbonyl linsidomine derivative (m/z 271) also only yielded a m/z 86 fragment.

Typical retention times for the linsidomine derivative were between 3.40 and 3.60 min. The total chromatography time of 4.5 min made it possible to analyse a large number of samples in a relative short period of time. The retention time for the linsidomine derivative had to be kept as short as possible to obtain peaks that were still above a signal to noise level of five. Longer retention times resulted in peaks that were tailing which resulted in higher limits of quantification, which were unacceptable for pharmacokinetic studies.

Fig. 2 shows representative chromatograms of calibration standards obtained at 27.41 and 0.70 ng ml^{-1} (LLOQ) and from a subject sample at 9.67 ng ml^{-1}.

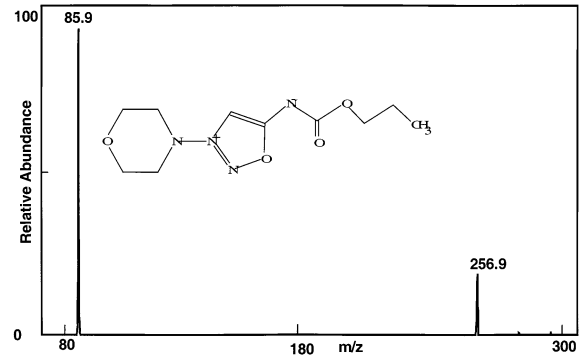

Fig. 1. MS of the molecular propoxycarbonyl linsidomine derivative ion (m/z 257) and the product ion formed at m/z 86 after collision (MS/MS).

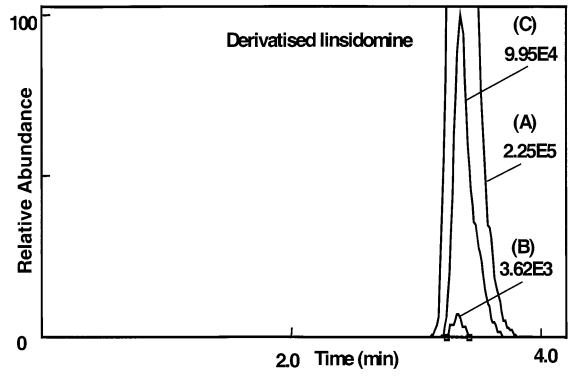

Fig. 2. HPLC of calibration standards containing 27.41 ng/ml (A) and 0.7 ng/ml (B) of linsidomine in plasma and of a subject sample containing 9.67 ng/ml (C) of linsidomine in plasma.

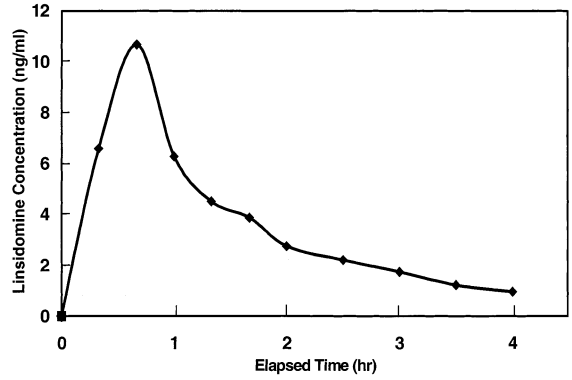

Fig. 3. Representative linsidomine plasma time profile as obtained after a single 4-mg oral dose.

In our initial development of an analytical method, we tried to extract linsidomine without derivatisation by different solid phase extraction cartridges, protein precipitation and different back-extractions. Most of these methods gave very low recoveries and underivatised linsidomine was unretained on different analytical columns causing it to co-elute with the injection peak, which in turn could lead to matrix effects. We therefore had to derivatise linsidomine with propyl chloroformate to yield the propoxycarbonyl linsidomine derivative before extraction, using the derivatisation and extraction method of Dutot et al. [3]. We could however not inject derivatised linsidomine in the back extracted HCl (0.01 M) onto the LCQ. The remaining derivatisation reagent after the first evaporation step resulted in problems with the ionisation of linsidomine, which made reconstituting the extract in mobile phase after the first evaporation step impossible. Back-extraction into different concentrations of formic acid gave inconsistent results with low recoveries. Back-extracting the linsidomine derivative out of the HCl (0.01 M) was therefore the only alternative. This was achieved by adding 0.5 M tris buffer and diethyl ether to the HCl (0.01 M) followed by a vortexing step. The diethyl ether containing the derivatised linsidomine could then be evaporated and the final residue dissolved in mobile phase. This method gave reproducible results without the use of an internal standard and there was no interference from remaining compounds in the extracts. The increased sensitivity of MS/MS detection compensated for the decrease in the absolute recovery, thereby allowing us to reach an acceptable LLOQ for pharmacokinetic studies. In this study, we only determined linsidomine but the method can be adapted for the simultaneous determination of molsidomine and linsidomine in plasma.

The method was employed to analyse plasma samples containing linsidomine after a single oral dose of 4 mg molsidomine in 30 healthy volunteers. Concentration versus time profiles were constructed for up to 10 h. The maximum plasma concentrations (C_{max}) obtained varied between 6 and 16 ng ml^{-1}. Fig. 3 shows a typical pharmacokinetic profile of a subject after receiving a 4 mg oral dose of molsidomine.

Although the extraction method is tedious, it is compensated for by the increased sensitivity, increased selectivity, the shorter chromatography time and the reproducibility of the analytical method.

4. Conclusion

A highly sensitive and selective method for the quantification of linsidomine in human plasma

has been developed and validated. Plasma concentrations of linsidomine could be quantified from 0.70 to 41.1 ng ml^{-1}. The higher specificity of LC used in tandem with MS enables the operator to use less chromatograhic separation, which results in shorter run times and a higher sample throughput. The study also showed how dependent electrospray ionisation can be on the amount and type of organic acid added to the mobile phase and how sensitive it could be to unwanted components remaining in the extracts.

References

[1] Y. Asahi, K. Shinndzaki, M. Nagaoka, Chem. Pharm. Bull. 19 (1971) 1079.
[2] D. Dell, J. Chamberlain, J. Chromatogr. 146 (1978) 465–472.
[3] C. Dutot, J. Moreau, P. Cordonnier, O. Spreux-Varoquax, C.J. Klein, J. Chromatogr. 528 (1990) 435–446.
[4] H. Koyama, T. Kubota, T. Yashiki, J. Takeda Res. Lab. 42 (1983) 52.
[5] W.D. Ollis, C.A. Ramsden, Adv. Heterocyclic Chem. 16 (1974) 1.
[6] C.D. Bevan, K. Modha, Internal Report Hoechst., 1981, KCM 1DI 40181, 10-2-1981.