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An LC–MS–MS method for the determination of cyclizine in human serum

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Abstract

Cyclizine is a piperazine derivative with anti-emetic activity that is useful in the prevention and treatment of nausea and vomiting associated with motion sickness. A liquid chromatography–tandem mass spectrometry (LC–MS–MS) method is presented for the quantitation of cyclizine in serum. Sample pretreatment involved liquid–liquid extraction of 200 μl of serum with dichloromethane after the addition of 100 μl each of ammonium hydroxide and internal standard solutions. The extracts were analyzed by HPLC on a Luna® C_{18} reversed-phase column and an ion-trap mass spectrometer with an electrospray interface. A limit of detection of 1 ng/ml was determined which allowed for the reliable measurement of cyclizine in the serum of human subjects. The method was found to be linear over the calibration range of 2.5–100 ng/ml. The applicability of this method was demonstrated by the analysis of serum obtained from a human volunteer following administration of a single 50 mg cyclizine hydrochloride tablet. The reported method was observed to have the necessary sensitivity, selectivity, precision and accuracy for monitoring cyclizine concentrations in human subjects following oral administration.

Keywords: LC–MS–MS; Cyclizine; Chlorcyclizine; Human serum

1. Introduction

Cyclizine (CYC), 1-(diphenylmethyl)-4-methylpiperazine or 1-benzhydryl-4-methylpiperazine [1] is a piperazine derivative that has been effectively used for the prevention and treatment of nausea and vomiting associated with motion sickness [2,3]. The structures of CYC and chlorcyclizine (CLCYC), the internal standard, are shown in Fig. 1.

The analysis of drugs in biological fluids requires an accurate, sensitive, selective and precise analytical technique to ensure a valid representation of the drug's absorption and disposition in the body.

Assay methods for CYC in both serum and urine have used colorimetric detection methods [4], derivatization with triti-

ated acetic anhydride [5], gas–liquid chromatography [6,7], GC–MS [8,9] and HPLC with ultraviolet detection [10,11]. Limitations of some of these methods include the lack of the requisite sensitivity and selectivity necessary for accurate assessment of the pharmacokinetics of the drug. In addition, large sample volumes and complex procedures such as the use of derivatization techniques result in the need to harvest inordinately large volumes of blood samples and tedious and extended sample preparation times. Subsequently an HPLC method with the requisite sensitivity using dual-electrode coulometric detection in the "oxidative-screen mode" was published in 1995 [12]. However, this method also requires a complex and time-consuming solid-phase extraction procedure followed by a micro phase-separation step to prepare samples for analysis. The current method has the requisite sensitivity, selectivity, accuracy and precision for the determination of CYC in serum, offers a rapid and simple sample pretreatment which requires only 200 μl of serum and includes an improved extraction procedure compared to those previously reported [12].

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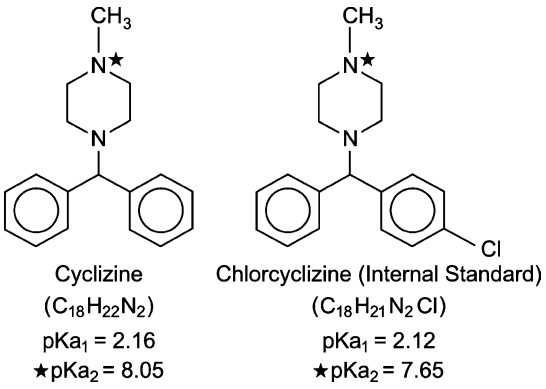

Fig. 1. Chemical structures of cyclizine (MW = 266.40) and chlorcyclizine (MW = 301.90).

In recent years, the use of LC–MS–MS in biomedical research [13] and more specifically for the quantitative determination of drugs in biological fluids [14] has increased exponentially. More recently a method for screening and quantitation of a range of antihistaminic drugs in blood by LC–MS has been published [15]. Whilst the sensitivity is comparable to that reported using HPLC-UV [11], the recovery of CYC was reported to be an unusually high value of 137%.

Development of techniques such as electrospray and atmospheric pressure chemical ionization (APCI) has largely contributed to the success of this technique, particularly in combination with HPLC and the application of LC–MS–MS with associated advantages of high specificity and greater sensitivity [16]. The advantages offered by LC–MS–MS coupled with a "micro-sample" liquid–liquid extraction method were therefore applied for the successful quantitative determination of CYC in human serum using CLCYC as an internal standard.

2. Experimental

2.1. Reagents and chemicals

CYC hydrochloride BP (CYC) was obtained from Lennon Ltd. Laboratories, South Africa and the internal standard, CLCYC hydrochloride (CLCYC) was obtained from the Wellcome Foundation Ltd., United Kingdom.

HPLC grade acetonitrile (Romil Ltd., Cambridge, United Kingdom), formic acid (99%) (Associated Chemical Enterprises, (Pty), Ltd., Johannesburg, South Africa) and aqueous ammonium hydroxide solution (25%, w/v) (Protea Laboratory Services, Port Elizabeth, South Africa) were of analytical reagent grade and were used without additional purification.

All other chemicals and reagents were of analytical grade and HPLC grade water was obtained by reverse osmosis and filtration through a Milli-Q system (Millipore, Milford, MA, USA) and was used to prepare all solutions.

Valoid® (GlaxoWellcome, South Africa) tablets having a label claim of 50 mg CYC as the hydrochloride which corresponds to 44 mg of CYC free-base, were purchased from a local pharmacy.

Drug free human serum was obtained from the South African blood transfusion service (Port Elizabeth, South Africa).

2.2. Equipment and LC–MS–MS conditions

A modular HPLC system consisting of a Spectraphysics Spectra System P2000 pump, an AS 3000 autosampler (Thermo Separation Products, San Jose, CA, USA) and a Finnigan Matt LCQ ion-trap mass spectrometer with an electrospray interface (San Jose, CA, USA) was used for the LC–MS–MS analysis.

Chromatographic separation was achieved using a Phenomenex, Luna® $C_{18}(2)$ 5 μm (150 mm × 2 mm), reversed-phase minibore analytical column (Torrance, CA, USA). The analysis was performed with a mobile phase consisting of 0.2% (v/v) formic acid in water (adjusted to pH 4 with ammonium hydroxide solution):acetonitrile (65:35, v/v) using a flow rate of 0.2 ml/min at 30 °C and an injection volume of 20 μl. An Upchurch Scientific Uptight Precolumn Kit (Oak Harbor, WA, USA) packed with glass beads, was used as a guard column.

Positive ion electrospray ionisation (ESI) mass spectrometry was performed on CYC and CLCYC. The MS parameters were optimised separately by direct infusion of a solution of each analyte (1 μg/ml) into the source at a rate of 5 μl/min, without splitting. The use of two segments during the chromatographic run made scanning at optimum conditions possible. The protonated molecular ions $[M+H]^+$ at *m/z* 267 (CYC) and *m/z* 301 (CLCYC) were initially monitored in the full scan mode between *m/z* 80 and *m/z* 350 using an isolation width of 5 amu for each ion. The HPLC eluate entered the mass spectrometer at a source voltage of 4.5 kV whilst the capillary temperature was maintained at 200 °C. The capillary voltage was set at 17 and 11.5 V for CYC and CLCYC, respectively, whilst the sheath to auxiliary gas ratio was set at 4:1. MS–MS experiments were subsequently undertaken through collision induced dissociation of the parent molecules (26% for CYC; 25% for CLCYC) and the resulting product ion was used for diagnostic confirmation.

2.3. Preparation of standard and quality control (QC) samples

Primary standard stock solutions of CYC (1 mg/ml) and CLCYC (1 mg/ml) as the hydrochloride salt, were prepared by dissolving an accurately weighed quantity of each compound equivalent to 20 mg of free base in water. In all cases, the solutions were protected from light using aluminium foil and stored at 4 °C during these studies. Serum standard (100 ng/ml) and QC (100 ng/ml) stock solutions were

prepared by spiking drug free serum with known aliquots of an appropriate dilution of an aqueous CYC solution (20 μg/ml). Serum calibration standard solutions over the concentration range from 2.5 to 100 ng/ml with the exception of the upper concentration (100 ng/ml) were prepared by spiking drug free serum with known aliquots of serum standard stock solution, followed by extraction. QC samples of 5, 20 and 80 ng/ml were prepared by spiking drug free serum with known aliquots of the QC stock solution of CYC (100 ng/ml). Aliquots (2 ml) of the QC samples and calibration standards were pipetted into 5 ml non-sterile polystyrene blue cap tubes and stored at −20 °C until required for use.

2.4. Extraction procedure

For the determination of cyclizine, 100 μl ammonium hydroxide (25%, v/v) and 100 μl aqueous internal standard solution (200 ng/ml) were added to two hundred microlitres (200 μl) of serum samples. The mixture was vortexed for 30 s after which 2 ml dichloromethane was added and vortexed for a further minute. The mixture was centrifuged at 6000 rpm for 3 min to separate the aqueous and organic layers. After removal of the organic layer the extraction was repeated on the residual aqueous layer.

The dichloromethane layers were pooled and dried at 40 °C under a gentle stream of nitrogen using an N-Evap® Model 112 analytical evaporator (Organomation Associates Inc., South Berlin, MA, USA). After drying, samples were reconstituted with100 μl of HPLC mobile phase, vortexed for 1 min and centrifuged at 6000 rpm, for 2 min and a 20 μl aliquot of the reconstituted sample was injected onto the chromatographic system.

2.5. Calibration and system validation

Calibration curves were constructed by plotting peak area ratios of CYC and internal standard against CYC concentration and analysis of linearity was performed, over three consecutive days, by a $1/\times$ weighted linear regression method using LCQAN®, a quantitation software package supplied with the mass spectrometer.

The standard curve for CYC incorporated 2.5, 5, 10, 25, 50 and 100 ng/ml with analysis of linearity from eight separate assays. The limits of quantitation (LOQ) for the assay was defined as the lowest concentration that could be measured with percent relative standard deviation (%R.S.D.) (precision) and percent relative error (%R.E.) (accuracy) of <20% as determined from spiked serum samples from eight separate assays.

The limits of detection (LOD) for the assay was defined as the lowest concentration of CYC that gave a signal-to-noise ratio of 3.

Reproducibility of the assay was assessed using the QC samples prepared as previously described in Section 2.3. A minimum of eight replicates per concentration were analyzed for both intra-assay and inter-assay precision, the latter being carried out on different days.

Recovery studies were carried out by adding internal standard at the reconstitution stage only, that is, the internal standard was not subjected to the extraction procedure. CYC recovery was determined at concentrations of 5, 20 and 80 ng/ml with five replicates at each concentration, by comparing the peak area ratios of CYC to CLCYC where the CLCYC was added after the extraction step, to the same area ratio for an equivalent aqueous sample (un-extracted). Recovery of the internal standard (CLCYC) was assessed in the same manner at a concentration of 200 ng/ml.

Stability of CYC in serum was determined from serum QC samples of 5, 20 and 80 ng/ml stored at 4 °C for 60 h. Post-extraction stability was determined by extracting QC samples at 5, 20 and 80 ng/ml and then left in the autosampler at ambient temperature for 24 h prior to LC–MS–MS analysis.

Freeze-thaw stability was tested with aliquots of spiked serum at concentrations of 5, 20 and 80 ng/ml, which were subjected to three freeze-thaw cycles prior to analysis.

2.6. Application of method

In order to assess the utility of this method for pharmacokinetic studies, a healthy volunteer was given a single Valoid® tablet following ethical approval by the Rhodes University Ethics Committee. Blood samples were collected at 2- and 4-h after administration. The blood was left to stand and the serum harvested for analysis using the method described and samples were extracted in duplicate.

3. Results and discussion

An LC–MS–MS assay was developed and validated to enable the quantitative analysis of CYC in serum samples.

The electrospray ionization of CYC and CLCYC produced the abundant protonated molecular ions $[M+H]^+$ at *m*/*z* 266.8 and 300.8, respectively, under positive ionization conditions, without any evidence of fragmentation. The full scan ESI mass spectrum of the protonated molecular ions of CYC and CLCYC is shown in Fig. 2. Collision induced dissociation of these ions resulted in the loss of the methylated piperazine moiety $[M-100]^+$ thereby producing fragment ions at *m*/*z* 167 and 201 for CYC and CLCYC, respectively (Fig. 3B and C). The quantification of the analytes was performed in MS–MS mode by monitoring both the protonated and product ions and interpolating the concentration from the peak area ratios (CYC/IS).

Examination of linearity over the concentration range 2.5–100 ng/ml yielded a slope of 0.0254, an intercept of 0.0225 and a correlation coefficient of 0.9994 from eight separate assays. The percent relative standard deviation (%R.S.D.) values ranged from 1.16 to 6.5%.

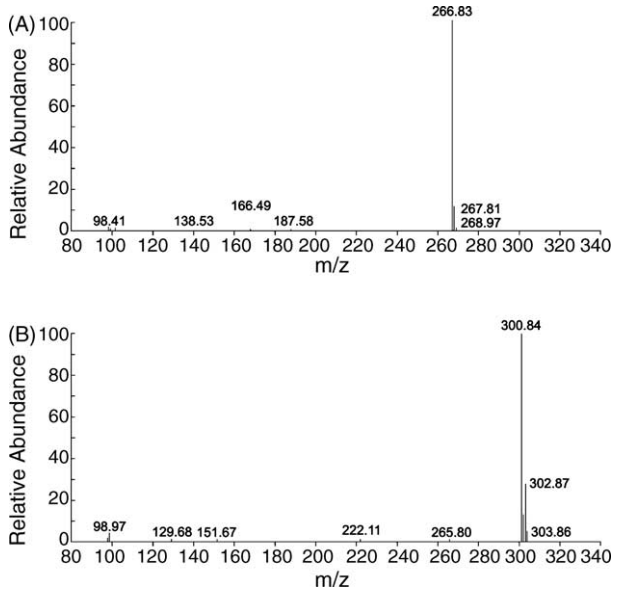

Fig. 2. Full scan mass spectrum showing the protonated molecular ion of (A) cyclizine and (B) chlorcyclizine (internal standard).

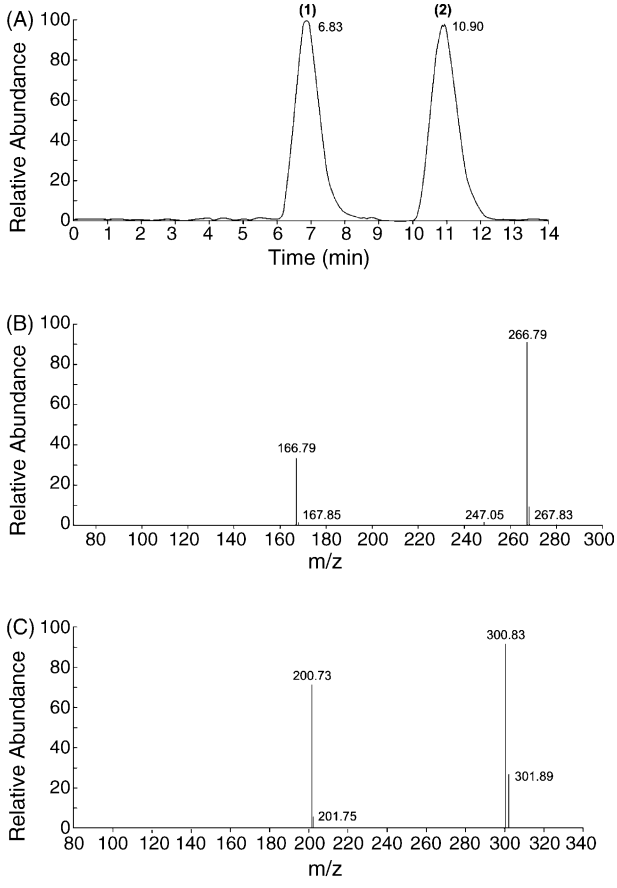

Fig. 3. (A) HPLC–MS–MS chromatogram of a serum sample obtained from a human volunteer 2-h after administration of a 50 mg cyclizine hydrochloride tablet showing cyclizine (6.83 min) and chlorcyclizine (10.9 min), (B) product ion mass spectrum of cyclizine, (C) product ion mass spectrum of chlorcyclizine.

Table 1
Intra- and inter-assay precision and accuracy data

Added (ng/ml)	Intra-assay precision ($n = 8$)			Inter-assay precision ($n = 8$)		
	Found	R.S.D. (%)	R.E. (%)	Found	R.S.D. (%)	R.E. (%)
5	4.4 ± 0.1	3.0	12.2	4.5 ± 0.2	4.8	11.0
20	20.4 ± 0.4	1.7	1.9	20.6 ± 1.0	5.1	3.2
80	85.0 ± 2.3	2.7	6.2	85.2 ± 3.0	3.5	6.5

Data expressed as mean ± S.D. for "found" values and mean data shown for %R.E.

The LOQ for the assay was 2.5 ng/ml with a corresponding %R.S.D. of 2.6 and a %R.E. of 12.2 and the LOD for CYC was 1 ng/ml.

Intra-assay precision yielded %R.S.D. values ranging from 3.0 at a concentration of 5 ng/ml to 2.7 at 80 ng/ml, with percent relative errors of 12.2, 1.9 and 6.2 at 5, 20 and 80 ng/ml respectively ($n = 8$). Percent R.S.D. values for the inter-assay precision studies were 4.8 at 5 ng/ml, 5.1 at 20 ng/ml and 3.5 at 80 ng/ml, with percent relative errors of 11.0, 3.2 and 6.5, respectively ($n = 8$) (Table 1).

CYC recovery rates were assessed by comparison of peak area ratios of aqueous standard solutions of CYC and samples to which internal standard was added after completion of the extraction ($n = 5$). The recovery rates were 74.7 ± 4.7% (mean ± S.D.), 86.3 ± 1.5 and 79.4 ± 1.9% with %R.S.D. values of 6.3, 1.7 and 2.1% at 5, 20 and 80 ng/ml, respectively, with an overall mean recovery of CYC 80.1 ± 2.8% (Table 2). The recovery of the internal standard, CLCYC (200 ng/ml) was 72.5 ± 1.6% (mean ± S.D.; $n = 5$).

Stability of the QC serum samples evaluated at 4 °C indicated that the drug was stable for a period of 60 h (Table 3). Post-extraction stability of samples left in the autosampler at room temperature indicated a loss of <10% at the 20 and 80 ng/ml concentrations and <15% at the 5 ng/ml concentration. Exposure of spiked serum samples to three freeze-thaw cycles prior to extraction had no significant effect on the

Table 2
Absolute recovery rates following extraction

Added (ng/ml)	Recovery (ng/ml)	R.S.D. (%)	Recovery (%)
5	3.7 ± 0.2	6.3	74.7 ± 4.7
20	17.3 ± 0.3	1.7	86.3 ± 1.5
80	63.5 ± 1.5	2.4	79.4 ± 1.9

Data obtained from five replicates at each concentration. Recovery data expressed as mean ± S.D.

Table 3
Stability of spiked QC serum samples stored at 4 °C for 60 h

Concentration (ng/ml)	R.S.D. (%)	Interpolated concentration	R.E. (%)
5	3.5	5.1 ± 0.2	2.7
20	1.5	22.1 ± 0.3	10.3
80	1.9	82.9 ± 1.6	3.7

Data obtained from five replicates at each concentration.

Table 4
CYC concentration following single oral dose administration

Time after administration (h)	R.S.D. (%)	Concentration (ng/ml) ($n = 5$)
2	4.3	36.7 ± 1.6
4	2.6	77.1 ± 2.0

Concentration data expressed as mean ± S.D. and mean data are shown for %R.S.D.

analysis of CYC where the %R.E. ranged from 8.8 at 5 ng/ml to <2 for the 20 and 80 ng/ml concentrations.

Analysis of serum samples obtained from a healthy volunteer who was administered a Valoid® tablet (50 mg CYC hydrochloride) after an overnight fast indicated that this assay is readily applicable for the quantitative analysis of cyclizine in serum samples.

Fig. 3A depicts a representative chromatogram obtained by LC–MS–MS analysis of a serum sample obtained from a human volunteer 2-h after oral administration of CYC. The analysis of blank serum samples did not show any interference at the retention times of CYC and CLCYC confirming the specificity of this method. Table 4 depicts the serum concentrations found in the subject at 2 and 4 h post administration.

Reported blood levels for CYC following intravenous administration of 50 mg CYC lactate to a single volunteer was approximately 300 ng/ml [6] and between 50 and 105 ng/ml in six human subjects following a 25 mg dose [17] and extraction of 1 ml aliquots of serum. The concentrations of CYC found in the serum of the subject used in this study, in which only a 200 μl sample was used are similar to those obtained in human studies in which the peak concentrations in plasma and serum after oral administration were 69 ng/ml [7] and 80 μg/ml using a 2 ml serum aliquot [8] and 57 ng/ml using a 1 ml sample [11].

The combination of tandem mass spectrometry with HPLC has proved extremely suitable for the highly specific quantitative and accurate measurement of CYC in serum.

An LC–MS–MS method [15] previously reported the quantitation of CYC with a LOQ of 5 ng/ml which was associated with a high recovery of 137%. This suggests that the LOQ was somewhat higher than 5 ng/ml possible due to the contribution of matrix effects.

4. Conclusion

The method reported in this paper describes a sensitive, selective and accurate LC–MS–MS method for the determination of CYC in human serum and offers a rapid and simple sample pretreatment requiring only 200 μl of serum compared to previously reported methods where 1 or even 2 ml samples were used. Clearly this implies that should lower concentrations need to be monitored, a larger sample volume could be used. Furthermore, the extraction procedure was more efficient than previously reported [12] and yielded reliable values [15].

The proposed method has the requisite sensitivity, accuracy and precision for the determination of CYC in serum and is readily applicable for bioavailability/bioequivalence and pharmacokinetic studies.

References

[1] S.A. Benezra, in: K. Florey (Ed.), Analytical Profiles of Drug Substances, vol. 6, Academic Press, New York, 1977, pp. 84–97.
[2] H.I. Chinn, W.R. Gammon, M.E. Frantz, J. Appl. Physiol. 5 (1953) 599.
[3] J.J. Brand, W.L.M. Perry, Pharmacol. Rev. 18 (1966) 895.
[4] R. Kuntzman, A. Klutch, I. Tsai, J.J. Burns, J. Pharmacol. Exp. Ther. 149 (1965) 29.
[5] R. Kuntzman, I. Tsai, J.J. Burns, J. Pharmacol. Exp. Ther. 158 (1967) 332.
[6] G. Land, K. Dean, A. Bye, J. Chromatogr. Biomed. Appl. 222 (1981) 134.
[7] D.S. Griffin, R.S. Baselt, J. Anal. Toxicol. 8 (1984) 97.
[8] R.V. Backer, P. McFeeley, N. Wohlenberg, J. Anal. Toxicol. 13 (1989) 308.
[9] M.C. Dumasia, L. Grainger, E. Houghton, Xenobiotica 32 (2002) 795.
[10] I. Kanfer, N.A. Sparrow, APhA Academy of Pharmaceutical Sciences Annual Meeting, Montreal, Canada, 1984.
[11] R.B. Walker, I. Kanfer, Chromatographia 24 (1987) 287.
[12] R.B. Walker, I. Kanfer, J. Chromatogr. Biomed. Appl. 672 (1995) 172.
[13] E. Gelpí, J. Chromatogr. A 1000 (2003) 567.
[14] E. Brewer, J. Henion, J. Pharm. Sci. 87 (1998) 395.
[15] M. Gergov, J.N. Robson, I. Ojanpera, O.P. Heinonen, E. Vuori, For. Sci. Int. 121 (2001) 108.
[16] F.M. Lagerwerf, W.D. van Dongen, R.J.J.M. Steenvoorden, Trends Anal. Chem. 19 (2000) 418.
[17] R.B. Walker, I. Kanfer, Eur. J. Pharm. Sci. 4 (1996) 301.