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Automated high-performance liquid chromatographic method for the analysis of two novel ergoline compounds in human plasma

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Abstract

A rapid and sensitive high-performance liquid chromatographic method for the determination of the novel ergoline derivatives sergolexole (compound I), its acid metabolite (compound II) and *cis-n*-(2-hydroxycyclopentyl)-6-methyl-1-(1-methylethyl)ergoline-8-carboxamide (LY215840, compound III) in human plasma is reported. The compounds were extracted from plasma by automated solid-phase extraction and analysed on a reversed-phase C_8 column with fluorescence detection. The limit of quantification for all compounds was 10 ng/ml and the response was linear over the range 10–1000 ng/ml. Validation studies showed the method to be both repeatable and reproducible with no interference from human plasma. The method has been used to support pharmacokinetic studies and has proved to be robust and effective.

Keywords: Ergolines; Sergolexole

1. Introduction

Serotonin is thought to play an important role in the pathophysiology of coronary heart disease. This is based on the evidence that serotonin can enhance platelet aggregation and induce coronary arterial vasoconstriction [1].

The effects of serotonin on both platelets and blood vessels result mainly from the activation of 5-HT_2 receptors. Consequently, 5-HT_2 receptor antagonists may prove effective in the treatment of coronary heart disease by attenuating the effects of serotonin on platelets and coronary arterial vasoconstriction.

Sergolexole (compound I) and *cis-n*-(2-hydroxycyclopentyl)-6-methyl-1-(1-methylethyl)ergoline-8-carboxamide (LY215840, compound III), (Fig. 1), are novel ergoline derivatives which have potent and selective activity as 5-HT_2 receptor antagonists [2,3]. The subsequent interest in these compounds as therapeutic agents for coronary heart disease required that a method be developed for their separation and quantification in human plasma samples from clinical trials. This method needed to be rapid, effective, sensitive, robust and cost effective.

Various methods are described in the literature for the determination of ergolines in plasma and urine. These include HPLC with fluorescence detection [4], HPLC with electrochemical detection [5] and HPLC coupled to atmospheric pressure ionisation mass spectrometry [6]. In all of these methods non-automated liquid extraction routines were used.

The present work describes the development and validation of a fully automated analytical method for

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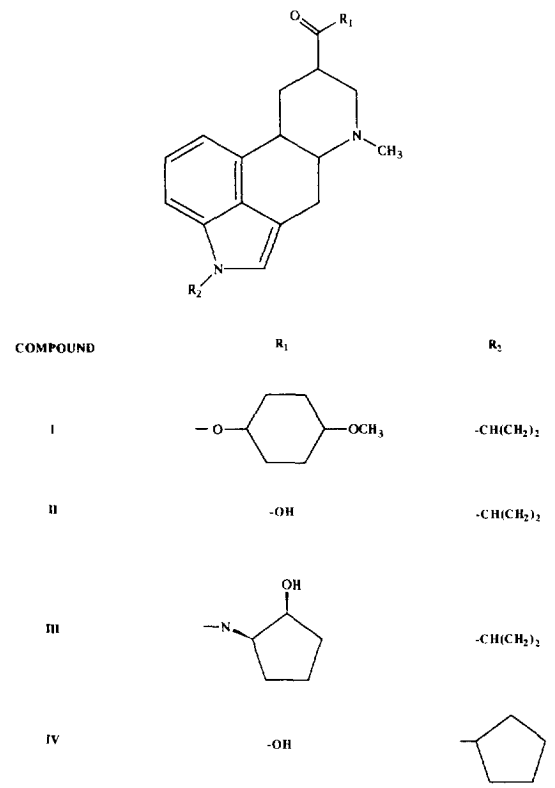

Fig. 1. Structures of the ergoline compounds.

the determination of compound I, its acid metabolite (compound II) and compound III in human plasma.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade solvents, acetonitrile, methanol and water were all obtained from Fisons (Loughborough, UK) or BDH (Poole, UK). Glacial acetic acid (Ar grade) was obtained from Fisons and ammonium acetate (analar grade) was obtained from BDH. Sergolexole maleate, compound II, the hydrochloride salt of compound III and the internal standard [*trans*-(8β)]-6-methyl-1-(cyclopentyl)ergoline-8-carboxylic acid (compound IV) were all obtained from Eli Lilly (Indianapolis, IN, USA). Control human plasma from a number of different donors was obtained from the UK Blood Transfusion Service (Edgware, UK).

2.2. Preparation of standards and controls

Separate 1 mg/ml stock solutions of compounds I, II, III and IV were prepared by dissolving the equivalent of 10 mg of free base for each compound in 10 ml of methanol. Calibration standards were prepared using a 10 μg/ml solution of compounds I and II and a 10 μg/ml solution of compound III diluted from the concentrated stocks in control human plasma. The standards for the assay of compound III were prepared by addition of the appropriate volumes of the 10 μg/ml solution of compound III to 50 ml of control human plasma. For the assay of compounds I and II the standards were prepared by addition of the appropriate volumes of the 10 μg/ml solution of compound I and II to 50 ml of control human plasma. Assay control samples were prepared in an identical manner to the calibration standards using fresh dilutions of the methanolic stock solutions.

All plasma standards and controls were stored frozen at −20°C in 1-ml aliquots until required. A working internal standard solution of 10 μg/ml was prepared by dilution of the 1 mg/ml stock solution of compound IV in methanol.

2.3. Sample preparation

Aliquots of each standard and control (1 ml) were allowed to thaw and were dispensed into 75×10 mm borosilicate glass culture tubes. Internal standard solution (25 μl) was added and the samples were vortex mixed. The samples were then extracted using Sep-pak plus custom C_8, 300 Å solid-phase extraction cartridges (Waters, Watford, UK). The C_8 solid-phase cartridges were first conditioned with 2 ml of methanol followed by 2 ml of water. The plasma samples were acidified by addition of either 100 μl of 10% acetic acid (compounds I and II) or 1 ml of 30 m*M* acetate buffer at pH 3.0 (compound III) and then loaded onto the C_8 cartridges. The cartridges were washed with 1 ml of water and compound I and II samples were then additionally washed with 0.5 ml of methanol–water (1:1, v/v). The remaining wash solutions were removed from the cartridge by purging with nitrogen. The samples were then eluted with 1.2 ml of methanol (compounds I and II) or 1.7 ml of methanol (compound

III) (approximately 0.2 ml remains on the cartridge) and 20 μl was injected onto the HPLC. The extraction and subsequent HPLC injection were performed automatically using a Millilab workstation (Waters).

2.4. Chromatographic instrumentation and conditions

The HPLC system consisted of a Merck-Hitachi L6200A Intelligent pump (Merck, Poole, UK), a Merck-Hitachi F-1050 fluorescence detector (Merck) and auto injection provided by a Millilab workstation. The analytical column (15 cm×4.6 mm) was packed with 5 μm Hichrom Base Deactivated C_8 (Hichrom, Reading, UK) and was protected by a 1 cm guard cartridge packed with the same material.

The mobile phase consisted of acetonitrile in 30 m*M* ammonium acetate (adjusted to pH 3.0 with glacial acetic acid). The ratio of acetonitrile to buffer was 36:64 (v/v) for the assay of compounds I and II and 29:71 (v/v) for the assay of compound III. The chromatography was performed at ambient temperature with a flow-rate of 1 ml/min. The analytes were detected by their fluorescence at an excitation wavelength of 290 nm and emission wavelength of 370 nm (compounds I and II) or 355 nm (compound III).

On-line data acquisition and subsequent calculations were carried out using a Hewlett-Packard HP1000 Model A90 computer operating with an in-house software package (Eli Lilly).

2.5. Determination of recovery, linearity, precision and accuracy

Control samples containing 10, 200 and 1600 ng/ml were prepared by diluting the appropriate volumes of the 10 μg/ml solution of compounds I and II or the 10 μg/ml solution of compound III with control human plasma. The 1600 ng/ml control was diluted 1:1 with control human plasma immediately prior to assay to give an actual control level of 800 ng/ml.

Six replicates of each control sample level were analysed on three separate days using duplicate calibration standards. Data from these experiments were used to establish linearity, accuracy, precision, sensitivity and specificity. Recovery was also determined by comparison of peak areas of extracted plasma standards with peak areas obtained from direct injection of methanolic standards of equivalent concentration.

3. Results

3.1. Precision, accuracy and sensitivity

The assays of compounds I, II and III were evaluated for precision and accuracy by replicate determinations of three plasma pools spiked with the analytes at three concentrations. Six replicates of the same pools were evaluated on three different days so that both intra- and inter-assay accuracy and precision could be determined.

Tables 1–3 show the precision and accuracy data for compounds I, II and III, respectively. The coefficient of variation (C.V.) and relative error (R.E.) of each of the three analytes for both intra- and inter-assay are within 10% at the two higher concentrations. The inter-assay R.E. and C.V. for each analyte at 10 ng/ml are within 10% and 15%, respectively. The intra-assay C.V. and R.E. are mostly within 15% and all are within 20% at 10 ng/ml, which was, therefore, taken as the limit of quantification.

Table 1
Coefficients of variation (C.V.) and relative errors (R.E.) for repeated analyses of three plasma pools containing compound I at different nominal concentrations

Day		Nominal concentration (ng/ml)		
		10.09	201.8	807.2
1	Mean (n=6)	10.03	198.6	790.9
	C.V.%	7.6	3.3	4.0
	R.E.%	−0.6	−1.6	−2.0
2	Mean (n=6)	11.53	213.25	810.1
	C.V.%	13.7	3.3	3.8
	R.E.%	14.3	5.7	0.36
3	Mean (n=6)	9.96	205.3	773.5
	C.V.%	18.4	4.7	5.1
	R.E.%	−1.3	1.7	−4.2
Inter-assay	Mean (n=18)	10.51	206.5	793.4
	C.V.%	14.9	5.0	4.7
	R.E.%	4.2	2.3	−1.7

Table 2
Coefficients of variation (C.V.) and relative errors (R.E.) for repeated analyses of three plasma pools containing compound II at different nominal concentrations

Day		Nominal concentration (ng/ml)		
		10.03	206	824
1	Mean (n=6)	11.7	205.6	811.8
	C.V.%	2.89	1.1	2.4
	R.E.%	13.6	−0.2	−1.5
2	Mean (n=6)	9.81	221.4	859.9
	C.V.%	8.24	2.4	3.2
	R.E.%	−4.8	7.5	4.3
3	Mean (n=6)	8.91	216	818
	C.V.%	10.6	6.6	5.6
	R.E.%	−13.5	4.8	−0.7
Inter-assay	Mean (n=18)	10.1	214.3	829.8
	C.V.%	13.7	5.0	4.5
	R.E.%	−1.6	4.0	0.7

3.2. Linearity

The linearity for each analyte was established over the range 10–1000 ng/ml using duplicate standards at concentrations of 10, 20, 50, 100, 200, 500 and 1000 ng/ml. In each case a correlation coefficient of greater than 0.99 was obtained.

Table 3
Coefficients of variation (C.V.) and relative errors (R.E.) for repeated analyses of three plasma pools containing compound III at different nominal concentrations

Day		Nominal concentration (ng/ml)		
		10	200	800
1	Mean (n=6)	9.5	187.7	792
	C.V.%	8.3	5.7	3.0
	R.E.%	−4.8	−6.2	−1.0
2	Mean (n=6)	10.0	180.0	793.0
	C.V.%	5.9	5.6	2.5
	R.E.%	0.0	9.8	−0.9
3	Mean (n=6)	12.0	192.0	782.5
	C.V.%	4.0	9.2	9.4
	R.E.%	20.0	−3.8	−2.2
Inter-assay	Mean (n=18)	10.5	186.8	789.2
	C.V.%	12.1	7.2	5.5
	R.E.%	5.2	−6.6	−1.4

3.3. Selectivity and recovery

Representative chromatograms of plasma extracts of compounds I, II and IV and of a control plasma blank are shown in Fig. 2. Good resolution was obtained between compound I, its acid metabolite (compound II), the internal standard (compound IV) and the endogenous plasma peaks. Fig. 3 shows representative chromatograms of plasma extracts of compound III and IV and of a control plasma blank. Good resolution was obtained between compound III, the internal standard (compound IV) and the endogenous plasma peaks.

The recovery of compounds I, II and III were determined to be 62%, 71%, and 74%, respectively, at a single concentration of 200 ng/ml.

3.4. Stability

The stability of compounds I, II and III was studied. All three compounds were found to be stable in human plasma stored at −20°C for up to three months.

4. Discussion

A method for the quantification of compound II in human plasma and urine following oral administration of compound I was developed at the Lilly Laboratory for Clinical Research, Indianapolis [7]. The method described in this paper evolved from modifications of that work.

The previous method was first modified to allow the simultaneous determination of compound I and compound II in a single extraction and chromatography procedure. The chromatographic procedure in the original method used a 22 cm×4.6 mm I.D. Spheri-5RP8 column with a close-coupled 3 cm guard column from Brownlee, together with a mobile phase of 30 m*M* ammonium acetate pH 3.0–acetonitrile (56:44, v/v). However, since retention times for compound I were very long with this system, an investigation of other column/mobile phase combi-

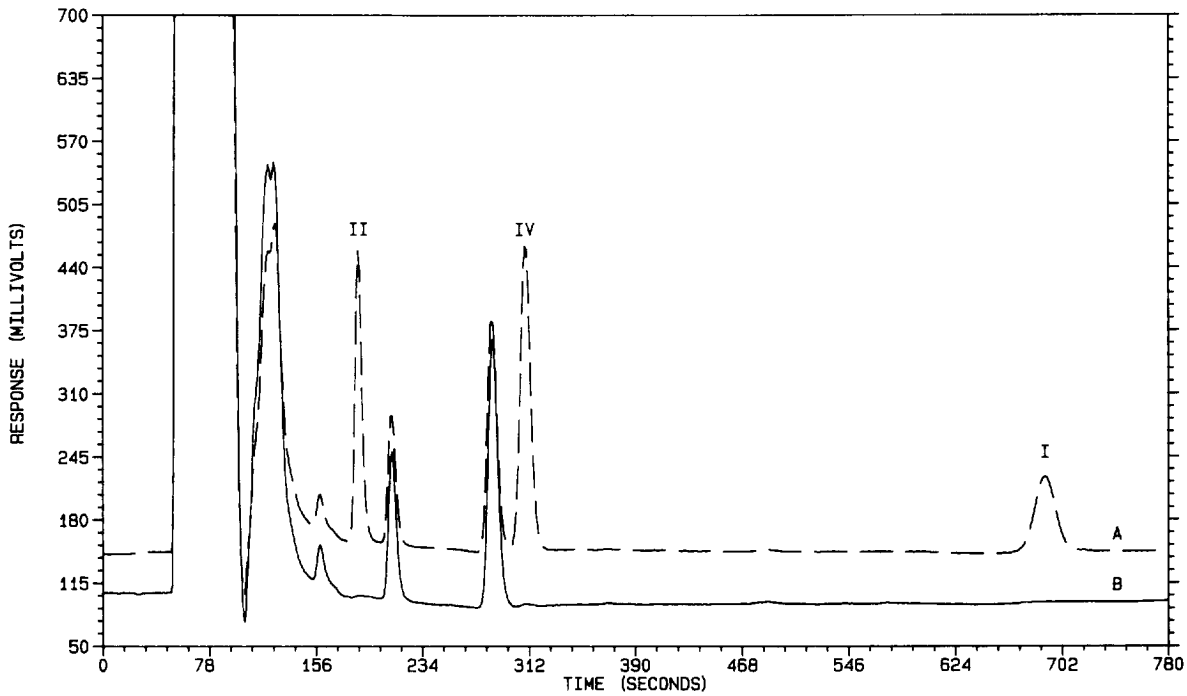

Fig. 2. Representative chromatograms of extracts of: (A) a 1 ml plasma standard containing compound I (100 ng/ml), compound II (100 ng/ml) and compound IV (250 ng/ml), and (B) 1 ml of blank plasma.

nations, gradient systems and different pH strengths was made. A 15 cm×4.6 mm I.D. column packed with Hichrom 5μ Base Deactivated C_8 together with a mobile phase composed of 36% acetonitrile in 30 m*M* ammonium acetate, pH 3.0, was found to provide suitable resolution of all components in a run time of approximately 13.5 min. Minor increases and decreases in pH were found to increase and decrease respectively the retention time of all components, enabling separation from endogenous interferences (Figs. 2 and 3). The extraction procedure was modified so that the injection of eluted samples was performed automatically by the Millilab workstation. This enabled samples to be extracted and analysed automatically in an overnight run.

Further modifications were made to the procedure to make it suitable for the analysis of compound III. The acetonitrile content of the mobile phase was reduced to 29% in order to resolve the compound III peak from an endogenous plasma peak. The extraction procedure was also modified for the determination of compound III. The 10% acetic acid used in the acidification step for the extraction of compounds I and II was found to cause precipitation of plasma proteins which gradually led to a blocked injector on the Millilab after many assays. Although this did not prevent routine use of this method for the assay of compounds I and II it was considered appropriate to modify this step for the assay of compound III. Consequently, 30 m*M* ammonium acetate buffer at pH 3.0 was used in place of 10% acetic acid for the extraction of compound III. This modification to the extraction procedure was subsequently shown to work equally well for compounds I and II, although it has not been validated in this case. The methanol–water wash step was removed because it reduced the extraction efficiency of compound III without significant benefit to sample clean-up. The elution volume was increased from 1.2 ml to 1.7 ml to further increase extraction efficiency. The emission wavelength of the fluorescence detector was changed from 370 nm to 355 nm after scans

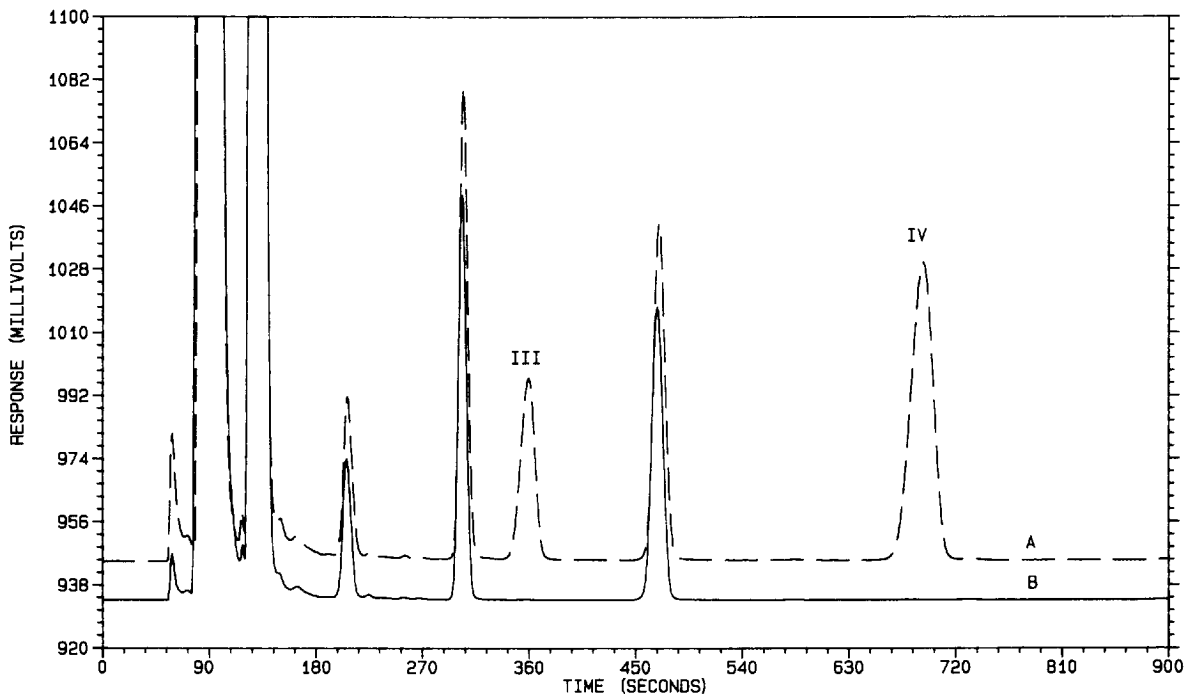

Fig. 3. Representative chromatograms of extracts of: (A) a 1-ml plasma standard containing compound III (100 ng/ml) and compound IV (250 ng/ml), and (B) 1 ml of blank plasma.

revealed this to be the optimum wavelength for compound III.

5. Conclusion

The method reported in this paper provides a rapid and sensitive method for the quantification of compounds I, II and III in human plasma.

The automated solid-phase extraction is more rapid and less labour intensive than the liquid extraction techniques generally used for the determination of ergolines. Fluorescence detection provided adequate sensitivity for these compounds and is more stable than electrochemical detection, as well as being a cheaper alternative to atmospheric pressure ionisation mass spectrometry.

Validation data shows the method to be accurate and precise (both intra-assay and inter-assay). The method has been successfully applied to obtain pharmacokinetic data from phase 1 clinical trials involving compounds I and III where it proved to be robust and effective.

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