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High-performance liquid chromatographic determination of the mucoregulatory drug CO/1408 in rat plasma and urine

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SUMMARY

A sensitive and selective high-performance liquid chromatographic (HPLC) method was developed for the determination of the mucoregulatory drug CO/1408 in plasma and urine. Samples containing an internal standard were prepared for analysis using a simple clean-up procedure based on Extrelut solid-phase extraction and chromatographed using a reversed-phase analytical column. Isocratic elution with a mobile phase consisting of 25 m*M* phosphate buffer (pH 2.5)–acetonitrile–methanol [85:10:5 or 87:9:4 (v/v) for plasma or urine analysis, respectively] was effected at a flow-rate of 0.8 ml/min. The eluate was monitored with an ultraviolet–visible variable-wavelength detector at 200 nm. The limit of quantification for the assay of CO/1408 was 80 ng/ml for plasma and 1 per 0.1 ml for urine samples. In spite of the high solubility of CO/1408 in water, the recovery from plasma and urine was very good and reproducible. The method was found to be applicable to pharmacokinetic studies of the drug in the rat.

INTRODUCTION

CO/1408, (−)-6(*S*)-hydroxy-4(*R*)-(1-hydroxy-1-methylethyl)-1-cyclohexene-1-ethanol (D, Fig. 1), is a synthetic mucoregulatory drug. Changes in mucociliary transport rate, modifications of rheological properties and biochemical composition of

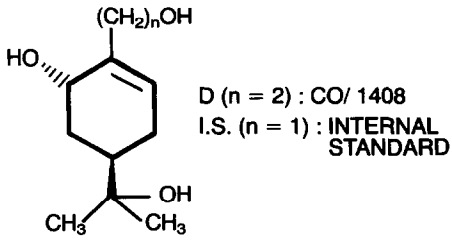

Fig. 1. Structures of CO/1408 and the internal standard.

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mucus have been studied in different animal species and are assumed to be involved in its action (unpublished results).

In order to investigate its pharmacokinetic profile in rats, a sensitive and selective assay for the determination of CO/1408 in plasma and urine was required. Taking into account the preliminary results of the biotransformation of this drug in rats (unpublished results), we have developed a high-performance liquid chromatographic (HPLC) method that is sufficiently rapid, reproducible, selective and suitable for the determination of the unchanged drug in rat plasma and urine after its administration at the active dose level.

EXPERIMENTAL

Chemicals and reagents

CO/1408 and the internal standard, (−)-6(*S*)-hydroxy-4(*R*)-(1-hydroxy-1-methylethyl)-1-cyclohexene-1-methanol (I.S., Fig. 1) were in-house reference standards (purity ⩾ 98.5%). Acetonitrile and methanol were of HPLC grade (Merck, Darmstadt, F.R.G.). All other chemicals were of analytical-reagent grade (Merck) and were used without further purification. Columns for extraction were prepared filling glass columns (13.5 × 1 cm I.D.) with about 1.2 g of granular support material (Extrelut, Merck), with a 1-cm round filter placed into the bottom of the column body.

Standard solutions

Stock standard solutions (1 mg/ml) of CO/1408 and I.S. were prepared in methanol. Working standards of CO/1408 (0.5, 1, 2, 3, 4, 10, 20, 30, 40, 50, 60, 80, 120, 160 and 200 μg/ml in water) and of I.S. (3, 12 and 50 μg/ml in water) were prepared from these stock solutions. The stock and working standard solutions were stored at −20°C and +4°C, respectively, and used within 1 month of preparation.

Chromatographic conditions

The HPLC apparatus consisted of a Model 5020 solvent metering instrument (Varian, Warrington, U.K.) and a Varian Model 2050 variable-wavelength UV detector operating at 200 nm with a sensitivity of 0.08 or 0.02 a.u.f.s. Samples (20 μl) were injected with an automatic injection system (WISP 710B; Waters Assoc., Milford, MA, U.S.A.) onto a guard column (20 mm × 4 mm I.D.) packed with Perisorb RP-8 (30–40 μm particle size) (Merck) in series with a Supelcosil LC-8 (5 μm) column (250 mm × 4.6 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.). Isocratic elution was performed at room temperature (24 ± 2°C) at a flow-rate of 0.8 ml/min. The mobile phase was 25 m*M* phosphate buffer (pH 2.5)–acetonitrile–methanol [85:10:5 and 87:9:4 (v/v) for plasma and urine assay, respectively]. Analyte peak heights were digitized and integrated using a Maxima 820 Chromatography Workstation (Waters Assoc.) running on an APC IV personal computer (NEC, Boxborough, MA, U.S.A.).

Extraction procedure

Plasma. Plasma samples (0.1–1 ml) were adjusted to 1.25 ml with distilled water and 0.25 ml of I.S. solution (3 or 10 μg/ml), 0.5 ml of 0.1 *M* glycine buffer (pH 2) and 0.75 g of sodium chloride were added. Each sample was vortex mixed for 2 min and

applied directly to an Extrelut column. After an equilibration period of 5–10 min, elution was carried out with 13 ml of chloroform–2-propanol (90:10, v/v). The eluate was collected and evaporated to dryness under a stream of nitrogen at 40°C in a water-bath. The residue was dissolved in 0.2–0.5 ml of the HPLC mobile phase and analysed.

Urine. Aliquots of 1 ml of biological samples, obtained by diluting urine with distilled water in the range 10–50-fold, were mixed with 0.25 ml of distilled water and 0.25 ml of I.S. solution (10 or 50 μg/ml), 0.5 ml of 0.1 *M* glycine buffer pH 2 and 0.75 g of sodium chloride were added. Each sample was vortex mixed and then processed as described for plasma.

Drug administration to rats

Male Wistar rats (180–200 g) were used for the preliminary kinetic study of the drug at the active dose level of 25 mg kg^{-1}. CO/1408 was administered both intravenously (bolus) and orally (by gavage) and animals were killed at various times over the following 24 h. Blood was collected from the abdominal artery using heparinized syringes and plasma was immediately separated. The total urine output was collected from each animal during 0–8, 8–24 and 24–48 h after administration. All samples were stored at −20°C until analysis.

RESULTS

Typical elution profiles from extracts of rat plasma and urine are shown in Figs. 2 and 3, respectively. The method provides good resolution of CO/1408 and I.S. from endogenous compounds in both plasma and urine samples. The method has been successfully applied to the determination of CO/1408 concentrations in plasma and urine after intravenous and oral administration of the drug to rats at an active dose of 25 mg/kg.

Stability

The stability of CO/1408 in rat plasma and urine, under the conditions chosen for sample storage until analysis, was investigated. Samples spiked at different concentrations (0.514, 1.028 and 5.140 μg/ml pre-dose plasma and 2.525, 5.050 and 10.100 μg per 0.1 ml pre-dose urine were prepared, stored at −20°C for 1 month, thawed and analysed together with freshly spiked samples. The results are reported in Table I. The plasma and urine standards were shown to be stable for at least 1 month, the deviations from the nominal value being $< 10\%$ in all samples.

Recovery

The recoveries of CO/1408 and I.S. were calculated by preparing plasma and urine samples at different concentrations (Table II). Six replicates of each plasma and urine standard were extracted and analysed. Recoveries were calculated by comparing the observed analyte concentrations with those obtained from the direct injection of unextracted standards containing the same amounts of CO/1408 and I.S. The average overall recoveries of CO/1408 and I.S. were 84% and 77% from plasma and 88% and 74% from urine, respectively. The recovery ratio of CO/1408 to I.S. was very reproducible in both plasma and urine.

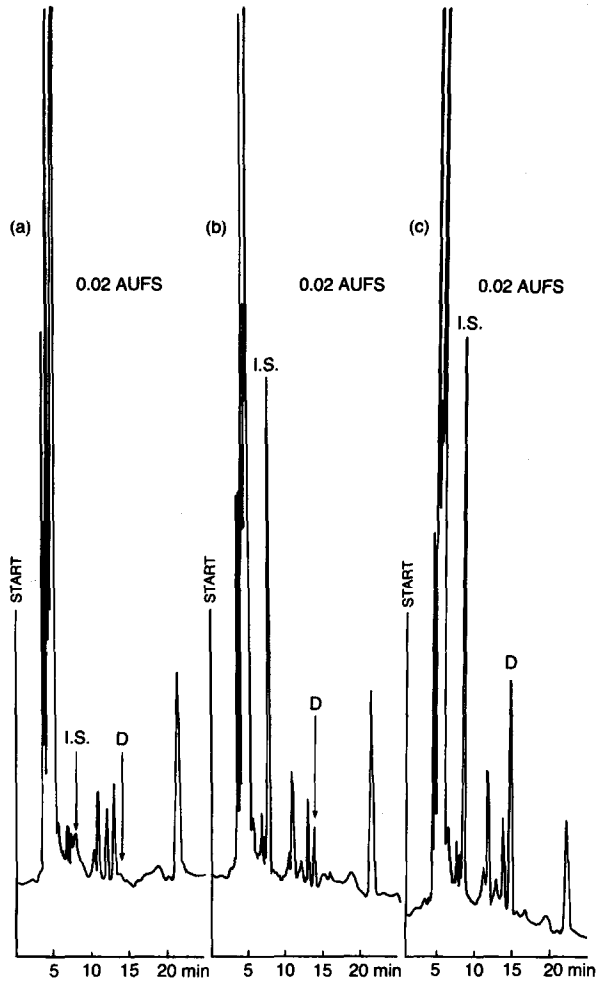

Fig. 2. Chromatogram of (a) a 1-ml plasma blank, (b) a 1-ml plasma blank spiked with 0.129 μg of CO/1408 and 0.787 μg of I.S. and (c) a 1-ml plasma sample containing 0.517 μg of CO/1408 10 h after oral administration of 25 mg kg^{-1} to a rat.

Calibration graphs

Calibration graphs were obtained using drug-free biological samples spiked with standard amounts of CO/1408 at concentration ranges expected to include the unknowns. Two plasma calibration graphs were used, one from 0.125 to 1 μg/ml of CO/1408 and 0.75 μg/ml of I.S. and the other from 1 to 10 μg/ml of CO/1408 and 3 μg/ml of I.S. Two urine calibration graphs were generated, one from 1 to 10 μg of CO/1408 and 3 μg of I.S. and the other from 10 to 50 μg of CO/1408 and 12.5 μg of I.S. Standards were extracted and analysed for each set of unknown samples. The standard daily responses were collected each day and cumulated with the previous graph to calculate the respective daily cumulative least-squares linear regression of the peak-height ratios of CO/1408 to I.S. *versus* drug concentrations[1].

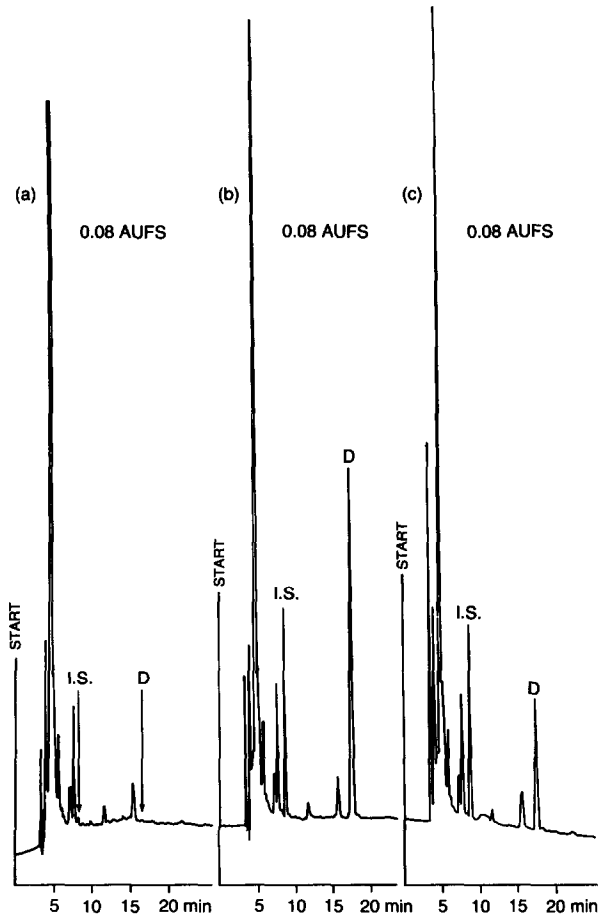

Fig. 3. Chromatograms of (a) a 0.1-ml urine blank, (b) a 0.1-ml urine blank spiked with 6.060 μg of CO/1408 and 2.79 μg of I.S. and (c) a 0.1-ml urine sample containing 2.468 μg of CO/1408 in the interval 8–24 h after oral administration of 25 mg kg^{-1} to a rat.

The analysis of variance (ANOVA) with an *F*-test ($\alpha = 0.05$) and lack of fit[2] were used to confirm the significance of the obtained regression and the adequacy of the linear model. CO/1408 concentrations in plasma and urine samples were calculated from the respective daily cumulative calibration graph[3].

An example of a day-by-day overall least-squares linear regression with the ANOVA, calculated in the drug concentration range 1–10 μg/ml in plasma, is reported in Table III.

Precision and accuracy

The intra-assay precision and accuracy were evaluated by measuring replicate spiked samples ($n = 6$ for each concentration of the drug used). The inter-assay precision and accuracy were calculated by analysing on each day, for five consecutive days, spiked samples ($n = 2$ every day for each concentration). The results are summarized in Table IV.

TABLE I

STABILITY OF CO/1408 IN RAT PLASMA AND URINE AT −20°C FOR 1 MONTH (n = 4)

Sample	*CO/1408 added (μg)*	*Mean CO/1408 observed (μg)*	*Intra-assay relative standard deviation[a] (%)*	*Deviation from amount added[b] (%)*
Plasma (1 ml)	0.514	0.502	4.2	−2.3
	1.028	0.960	3.7	−6.6
	5.140	4.985	5.7	−3.0
Urine (0.1 ml)	2.525	2.533	9.1	+0.3
	5.050	5.070	3.0	+0.4
	10.100	10.648	2.6	+5.4

[a] Relative standard deviation calculated as $(s/\bar{x}) \cdot 100$, where $\bar{x}$ is the mean amount found and s is its standard deviation.

[b] Calculated as $[(\bar{x}-\mu)/\mu] \cdot 100$, where μ is the amount added.

TABLE II

RECOVERIES OF CO/1408 AND INTERNAL STANDARD FROM PLASMA AND URINE (n = 6)

Sample	*CO/1408 added (μg)*	*I.S. added (μg)*	*Recovery (mean ± S.D.) (%)*		*CO/1408/I.S. recovery ratio (mean ± S.D.)*
			CO/1408	*I.S.*	
Plasma (1 ml)	0.514	0.787	81.79 ± 4.8	76.33 ± 4.5	1.071 ± 0.034
	1.028	0.787	77.45 ± 3.2	72.65 ± 4.7	1.066 ± 0.043
	2.570	5.245	84.26 ± 6.1	75.82 ± 5.9	1.111 ± 0.128
	7.710	5.245	90.87 ± 10.1	83.12 ± 9.4	1.093 ± 0.092
Urine (0.1 ml)	3.030	2.790	89.63 ± 6.6	77.25 ± 3.9	1.160 ± 0.046
	6.060	2.790	87.82 ± 5.5	72.86 ± 6.7	1.208 ± 0.049
	12.120	2.790	86.54 ± 8.7	72.81 ± 8.5	1.191 ± 0.030

TABLE III

DAY-BY-DAY CUMULATIVE REGRESSION LINE WITH THE RESPECTIVE ANALYSIS OF VARIANCE (ANOVA)

Day	*n[a]*	*Slope*	*y-Intercept*	*Correlation coefficient*	F_{ratio}	
					ANOVA[b]	*Lack of fit[c]*
1	10	0.198	−0.021	0.9990	4060.673	0.476
2	13 (10+3)	0.197	−0.004	0.9990	5438.352	0.662
3	16 (13+3)	0.197	−0.007	0.9989	6609.719	1.428
4	19 (16+3)	0.195	−0.003	0.9985	5525.609	0.866

[a] Number of standards (previous standards + daily cumulant) of the regression line.

[b] Regression is significant, F_{ratio} is always greater than the tabulated F value.

[c] Lack of fit is not significant, F_{ratio} is always less than the tabulated F value.

TABLE IV

INTER- AND INTRA-ASSAY PRECISION AND ACCURACY OF THE DETERMINATION OF CO/1408 IN RAT PLASMA AND URINE

Sample	*Amount added (μg)*	*Intra-assay*		*Inter-assay*	
		Relative standard deviation[a] *(%)*	*Accuracy*[b] *(%)*	*Relative standard deviation*[c] *(%)*	*Accuracy*[d] *(%)*
Plasma (1ml)	0.257	6.1	−2.7	4.7	+2.9
	0.771	1.9	−2.1	2.6	+0.8
	2.570	3.3	+1.0	2.9	+0.6
	7.710	1.8	+1.3	1.5	+1.5
Urine (0.1 ml)	3.030	2.5	−2.6	7.6	−2.4
	6.060	0.6	+1.9	2.4	+2.9
	12.120	1.0	−0.9	3.5	+1.3

[a] Calculated as $(s/\bar{x}) \cdot 100$, where $\bar{x}$ is the mean amount found ($n = 6$) and s is its standard deviation.

[b] Calculated as $[(\bar{x}-\mu)/\mu] \cdot 100$, where μ is the amount added.

[c] Calculated as in the first footnote but $\bar{x}$ is the between-days mean amount found ($n = 10$) and s is its standard deviation.

[d] See the second and third footnotes.

All relative standard deviations for the intra- en inter-assay precision were below 6.5% in plasma and 8% in urine. The intra- and inter-assay accuracies were also found to be good; the observed means ranged from (−)2.7 to 2.9% in plasma and from (−)2.6 to 2.9% in urine.

Limit of quantification

The limit of quantification (LOQ) was defined as the amount of CO/1408 per ml of plasma or per 0.1 ml of urine giving a signal-to-noise ratio of 10 (ref. 4). The LOQ in plasma was 80 ng/ml and that in urine was 1 μg per 0.1 ml.

DISCUSSION

Owing to the high water solubility of CO/1408 (42%, w/v), we have not been able to develop a reproducible and efficient procedure for the liquid–liquid extraction of this compound from biological media. As an alternative, a number of different types of solid-phase extraction columns and eluting systems have been investigated. Of the many phases tested, only the Extrelut type yielded good recoveries when the biological medium was saturated with sodium chloride and the column was eluted with chloroform–2-propanol (90:10, v/v). Further, the biological sample was sufficiently purified to permit the resolution of CO/1408 and the I.S. from endogenous plasma and urine components during HPLC at 200 nm. We selected HPLC with UV detection even though the products shows only UV end absorption, because degradation of CO/1408 under gas chromatographic (GC) conditions precluded GC analysis of the drug without derivatization. On the other hand, the HPLC analysis was sufficiently rapid; using isocratic elution with a mobile phase buffered to pH 2.5 the

retention times for CO/1408 and the I.S. were 7.2 and 13 min for plasma and 8.5 and 17.5 min for urine, respectively, with a total analysis time for each sample of 20 min.

In addition, this HPLC method yielded a limit of quantification for CO/1408 as low as 80 ng when using 1 ml of plasma or 1 μg when using 0.1 ml of urine. Therefore, this method is sufficiently selective, rapid, sensitive, precise and accurate to be applicable to pharmacokinetic studies of the drug in rats.

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