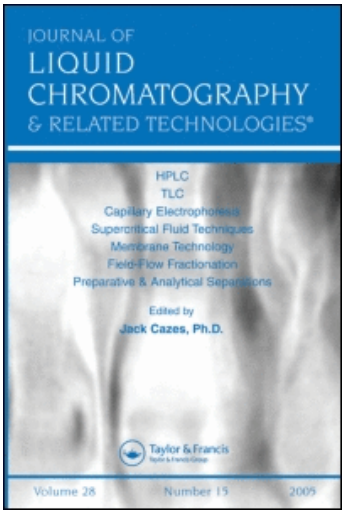

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A Sensitive High Performance Liquid Chromatographic Determination of Clopamide in Human Plasma

Sompon Wanwimolruk[a]
[a] Department of Pharmacy, University of Otago Medical School, Dunedin, New Zealand

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A SENSITIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CLOPAMIDE IN HUMAN PLASMA

SOMPON WANWIMOLRUK
Department of Pharmacy
University of Otago Medical School
P.O. Box 913
Dunedin, New Zealand

ABSTRACT

A simple and sensitive high-performance liquid chromatographic method for quantitation of clopamide in human plasma has been developed. The assay uses a reversed-phase C18 microbore column (2 mm I.D. x 100 mm) packed with 5 μm ODS Hypersil. The chromatographic separation was achieved by using an isocratic mobile phase comprising acetonitrile-10 mM phosphate buffer pH 4 (17:83, v/v) at a flow rate of 0.5 ml/min. The eluant was monitored by a UV detector operating at 241 nm. The assay was based on an organic extraction before chromatographic separation. To 1 ml plasma sample, 100 μl of the internal standard, methylparaben (300 ng/ml), and 8 ml of diethyl ether were added. The samples were shaken and centrifuged, the organic layer was then transferred to a tapered centrifuge tube and evaporated to dryness. The residue was reconstituted and injected onto the HPLC column. The inter-and intra-assay coefficients of variation were found to be less than 10%. The lowest limit of detection for clopamide in plasma was 5 ng/ml. The method is sensitive, specific and allows for routine analysis in the pharmacokinetic studies.

INTRODUCTION

Clopamide is an oral diuretic agent which has been proved to be effective in the treatment of hypertension and cardiac failure at a recommended daily dose of 5-20

mg/day (1-4). Combinations of clopamide (5 mg) and pindolol (10 mg) have also become popular for antihypertensive therapy especially if the desired drop in blood pressure does not occur with the diuretic alone (5). Pharmacokinetic and clinical trials of clopamide have been limited by the consequence of low therapeutic daily doses used and the lack of a sensitive assay technique. Two analytical methods are available for the determination of clopamide in human plasma using either gas-liquid chromatography-mass spectroscopy (6) or HPLC (4) assays. In the present study a simple and sensitive HPLC method is described. This procedure is suitable for quantitating clopamide in plasma at concentrations as low as 5 ng/ml.

MATERIALS AND METHODS

Reagents and Chemicals

All chemicals were of analytical grade. Clopamide was kindly supplied by Pacific Pharmaceutics Ltd (Auckland, New Zealand). Methylparaben was purchased from Sigma Chemicals Co (St Louis, MO, USA). HPLC-grade acetonitrile and diethyl ether were obtained from BDH Ltd (Poole, UK). Water was double glass distilled and MilliQ® filtered. All glassware was cleaned and silanized with 0.5% Aquasil® (Pierce, Rockford, IL, USA) before use.

Standard Solutions and Internal Standard

A stock solution containing 50 μg/ml of clopamide was prepared in methanol. The internal standard stock solution of methylparaben (30 μg/ml) was also prepared in methanol. The solutions were stored at -20°C until required and renewed every week. Plasma standard solutions of clopamide for the calibration curves were prepared by appropriate dilution of the stock clopamide solutions with drug-free plasma so that concentrations of 5, 10, 25, 50, 100, 250, 350 and 500 ng/ml were obtained. The internal standard solution of methylparaben (300 ng/ml) was prepared fresh each day of analysis by dilution of the stock solution with distilled water.

Sample Preparation

To 1 ml of plasma in a silanized centrifuge tube, 100 μl of the internal standard (300 ng/ml methylparaben) was added. The contents were extracted with 8 ml diethyl ether on a mechanical shaker for 10 minutes and briefly centrifuged (3000 g, 4°C) for 5 min. The organic layer was transferred to a clean tapered centrifuge tube

and evaporated to dryness under vacuum centrifugation (SpeedVac Concentrator, Savant Instruments Inc, NY, USA) with heating operated at 40°C. The dried residue was reconstituted with 150 µl of mobile phase and a sample (70 µl) was injected on to the HPLC column. The injection of sample up to a volume of 100 µl on to the column did not cause peak broadening. There was a good linear relationship between the peak heights and the injection volume ($r = 0.989$, $p < 0.01$).

Calibration Curve

Peak heights for clopamide and the internal standard (methylparaben) were measured and peak height ratios (clopamide/internal standard) were used for preparation of a calibration curve. The calibration curves were constructed by plotting plasma clopamide concentrations (x axis), expressed as ng/ml, versus peak height ratios (y axis), using linear regression.

Chromatographic Conditions

The HPLC system consisted of a Model 250 Perkin Elmer LC pump (Perkin Elmer Corporation, Norwalk, CT, USA) equipped with a WISP 712 Waters autoinjector (Waters Asso., Milford, MA, USA). A variable wavelength ultraviolet detector (Spectroflow 757, Kratos Analytical Instruments, Ramsey, NJ, USA) was used. The detector wavelength was set at 241 nm with an attenuation of 0.1 a.u.f.s. The chromatographic response was recorded by a Hitachi D2500 integrator (Hitachi, Tokyo, Japan) setting with an attenuation of 3 (i.e. 8 mV). A microbore HPLC column (2 mm I.D. x 100 mm) packed with a reversed-phase C18 material, 5 µm ODS Hypersil (Shandon, London, UK) was used. A guard column was not used. The average column life time was approximately 400 injections with extensive washing with methanol-water (50:50, v/v) after each run. Analysis of the samples of clopamide was performed using a mobile phase consisting of an acetonitrile - 10 mM disodium hydrogenphosphate buffer, pH 4 mixture (17:83, v/v). The flow rate was 0.5 ml/min. Chromatographic separations were performed at room temperature.

RESULTS AND DISCUSSION

Chromatographic separation of clopamide and the internal standard was achieved by using a mobile phase consisting of an acetonitrile - 10 mM disodium

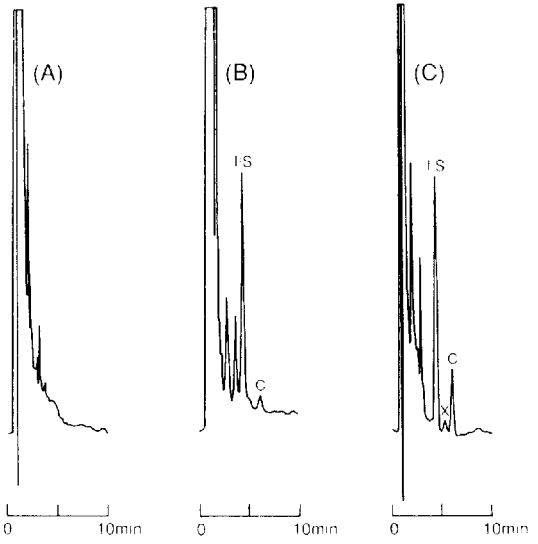

FIGURE 1

Typical chromatograms of extracts of human plasma: (A) blank plasma; (B) plasma spiked with 5 ng/ml clopamide; and (C) plasma with 22 ng/ml clopamide from a healthy subject 16 hours after an oral dose of 15 mg clopamide as three combined pindolol-clopamide tablets (Viskaldix, Sandoz). Peaks: I/S = internal standard (methylparaben); x = plasma endogenous peak; C = clopamide.

hydrogenphosphate buffer, pH 4 (17:83, v/v). This provides a good resolution between the internal standard and clopamide and there was no endogenous interference with the peaks of interest. The retention times for the internal standard and clopamide were 4.2 and 5.9 min. Figure 1 shows the chromatograms for blank plasma, plasma spiked with 5 ng/ml of clopamide and human plasma obtained 16 hours after drug administration. Blank plasma samples from 25 subjects were analysed and no plasma endogenous peaks co-eluting with the internal standard and clopamide were detected. Some of these blank plasma (9/25) had an endogenous peak appearing at 5.1 min which was between the internal standard and clopamide peaks.

TABLE 1

Within-run Reproducibility of the Assay for Clopamide in Plasma

Nominal Concentration (ng/ml)	n	Observed Concentration[1] (ng/ml)	C.V. (%)	Accuracy[2] (%)
5	6	4.93 ± 0.20	4.1	98.6
100	5	101 ± 3	3.3	101.5
500	5	506 ± 22	4.3	101.2

[1] Results given are mean ± S.D.

[2] Accuracy (%) = $\frac{\text{observed concentration}}{\text{nominal concentration}} \times 100$

Recovery of clopamide from plasma was estimated by comparing the peak heights of clopamide and the internal standard with those obtained by direct injection of the pure standards of clopamide and the internal standard. The mean recovery for clopamide (n = 3) from plasma sample was 81.7 ± 6.9% (S.D.) at 10 ng/ml and 82.5 ± 4.9% at 100 ng/ml. The absolute recovery for the internal standard was 85.9 ± 8.3% (n = 3). The recovery of clopamide from plasma was not improved by acidification of plasma samples with 100 µl of 2 N HCl (final sample pH = 1.5). Alkalinisation of plasma samples with 100 µl of 4 N NaOH (final sample pH = 12.5) produced an interfering peak with the internal standard. The extraction of sample with ether was found to achieve a maximal recovery after shaking on the mechanical shaker for at least 10 minutes.

The calibration curve for clopamide was linear over the concentration range of 5 to 500 ng/ml with the square of correlation coefficient (r^2) greater than 0.99. The typical linear relationship for the calibration curve can be expressed by the equation : $y = 0.01457x$. The intercepts (a) in all calibration curves were found to be statistically insignificant ($p > 0.05$) and were thus not included for the calculations. The day-to-day coefficient of variation (C.V.) of the slope of the calibration curves of clopamide was 5.4% (n = 12).

The within-run (within-day) reproducibility and precision of the assay together with the C.V. is shown in Table 1. At all concentrations the C.V. was less than 10%. The results indicate good precision of the assay. The reproducibility result at

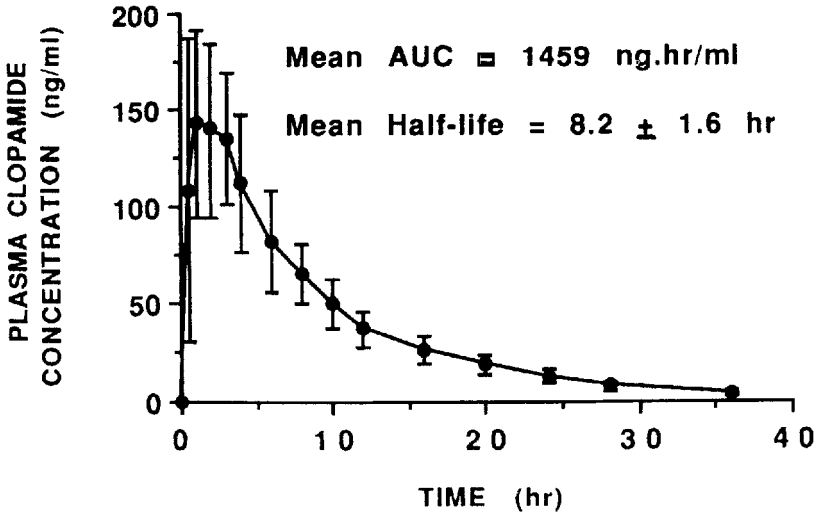

FIGURE 2

Mean plasma concentrations of clopamide versus time in 18 healthy subjects after an oral administration of 15 mg clopamide as three combined pindolol-clopamide tablets (Viskaldix, Sandoz). The vertical cross-hatched bars represent the standard deviation. AUC is area under the plasma clopamide concentration-time curve.

a concentration of 5 ng/ml gave mean and S.D. for the observed value of 4.93 ± 0.20 ng/ml with a C.V. of 4.1%. This C.V. is much lower than the generally accepted C.V. of 20% for minimum quantifiable concentration (MQC). A typical chromatogram of 5 ng/ml plasma clopamide standard is presented in Figure 1 (B). Thus the MQC or the detection limit of sensitivity for this assay was assigned at 5 ng/ml.

Plasma samples stored at - 70°C for up to 2 months showed no signs of decomposition and practically the same concentration values were obtained (n = 6). This suggests that clopamide is stable under these storage conditions for at least 2 months.

Possible interference by other drugs at their therapeutic concentrations was evaluated. Commonly used drugs tested not interfering with the assay included aspirin, salicylic acid, paracetamol, atenolol, labetalol, metoprolol, propranolol,

chlorothiazide, cyclopenthiazide, frusemide, amiloride, indapamide, diazepam, lorazepam, triazolam, phenytoin, valproate, carbamazepine, digoxin, quinidine, diltiazem verapamil and theophylline. Under the chromatographic conditions described, pindolol was eluted with a retention time of 5.3 min close to the clopamide peak. Although pindolol and clopamide were not well resolved chromatographically from each other, the blank plasma sample spiked with a high concentration (300 ng/ml) of pindolol extracted by the procedure used for this assay showed no interference peak with clopamide. This is because pindolol is a basic drug with pK_a of 8.8 thus being essentially ionised at the physiological plasma pH and not extracted. Therefore, the assay described here is also applicable to determining plasma clopamide concentration in the presence of pindolol. For example, in the analysis of clopamide from subjects or patients who have taken a combination tablet of clopamide and pindolol. In addition, attempts have been made to improve the resolution between clopamide and pindolol peaks by adjusting the strength of the organic modifier, acetonitrile (10 and 13 %), and pH (pH 2 and 6). These changes had no significant effect on the resolution of these two drugs. As temperature control facilities were not available, no tests were conducted to determine whether modification of the column temperature would obviate this problem.

Plasma samples (over 400 samples) from healthy subjects participating in a bioavailability study were analysed by this method. The mean plasma clopamide concentration-time profile observed in 18 subjects after an oral dose of 15 mg clopamide are shown in Figure 2. The mean peak plasma concentrations of 185.7 ± 62.1 ng/ml was reached 1.8 ± 1.0 hours after drug administration. After 36 hours the majority of the subjects still showed plasma clopamide concentrations above the assay sensitivity limit. The mean elimination half-life of clopamide was 8.2 ± 1.6 hours. These data are in agreement with those reported in previous studies (4,6).

In summary, the method described in this study is simple, sensitive and shows a good reproducibility. This procedure has been shown to be suitable for routine use in pharmacokinetic studies and drug monitoring in patients.

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