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Short Communication

Rapid determination of the metabolic pool of ethyl loflazepate in biological fluids by capillary gas chromatography

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Introduction

Ethyl loflazepate (Victan) is a 1,4 benzodiazepine that is used as an anxiolytic agent [1]. Metabolism studies showed that ethyl loflazepate was transformed to loflazepate (metabolite M1) in the intestinal wall [2]. It is known that M1 is decarboxylated to form descarboxy loflazepate (M2) during sampling, storage and extraction of biological fluids [2, 3]. Compound M2 is a common metabolite of other benzodiazepines such as flurazepam and quazepam.

In the case of flurazepam, several methods have been proposed in the literature for the determination of metabolite M2. These methods used different techniques such as GLC [4–10], GLC coupled with MS [11], spectrophotofluorimetry [12] or differential pulse polarography [13]. A GLC method has been published for quazepam and its metabolites [14]. There are few published reports on the determination of ethyl loflazepate; GC–MS [2] and GLC [3] assays have been proposed. However, in all the methods described, the extraction step was too long; thus those methods were not suitable for routine analysis. A HPLC method with on-line radioactivity detection and direct injection of the crude plasma has also been developed for metabolic studies in animals [15]. Several authors [2–6, 8–11, 14] used packed columns for GLC, but a satisfactory specificity was not attained; thus it was not possible to use such methods in those patients with hepatic or renal failure who were receiving concurrent medication.

The present work describes a capillary GLC method that permits rapid determination of compound M2 in the various biological fluids; the sensitivity and specificity are significantly better than those of previously published methods.

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Experimental

Chromatographic analysis

The analysis was performed on a Varian model 3700 GLC system equipped with a glass moving-needle injector and an electron capture detector.

The carrier gas was helium N 55 with a head-column pressure of 0.5 bar; the auxiliary gas was argon–methane (90:10, v/v) with a flow rate of about 20 ml min^{-1}. The temperature of the injection port and of the detector was 300°C. The retention times for the final M2 metabolite and its internal standard, delorazepam, were 5.9 and 7.9 min, respectively, at an oven temperature of 275°C (Fig. 1).

Capillary columns. The columns were glass wall-coated superior capacity open tubular (WSCOT) capillary columns (25 m × 0.50 mm i.d.) with an apolar silicone stationary phase (OV-1) of film thickness 1 μm.

Standard solutions. The internal standard solution was prepared by accurately weighing out 10 mg of delorazepam into a 10-ml volumetric flask and dissolving this substance in acetone–hexane (20:80, v/v). Serial dilutions were also prepared in this acetone–hexane mixture. Standard solutions of loflazepate were prepared in buffer (pH 10) obtained by adjusting 0.1 M potassium bicarbonate with potassium hydroxide. Serial dilutions were also prepared in buffer (pH 10).

Extraction procedure. A volume of 0.5 ml of plasma and 0.1 ml of 0.5 M sulphuric acid (H_2SO_4 Ulrex) were added to a suitable volume of the internal standard solution that had been evaporated to dryness in tapered 10-ml tubes under a stream of nitrogen; the mixture was extracted for 5 min with 1 ml of butyl acetate. The tubes were gently shaken on an Ika Vibrax VXR shaker at 1600 vibrations per min to avoid serious problems

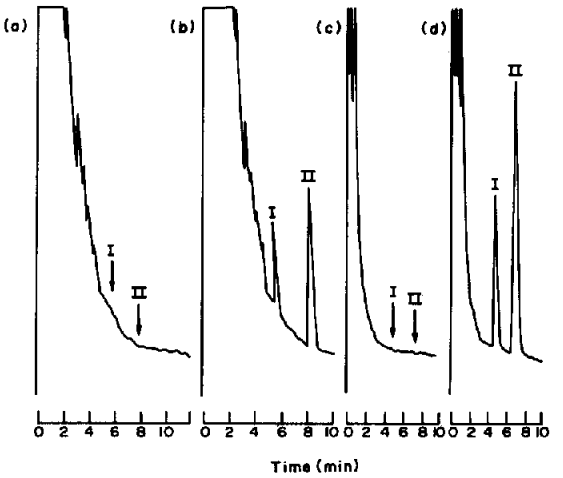

Figure 1
Typical chromatograms obtained for: (A) control plasma; (B) control plasma spiked with 0.5 ng ml^{-1} M1 (I.S. = 0.1 ng); (C) plasma from a subject before administration of ethyl loflazepate; and (D) plasma from the same subject 1 h after oral administration of 4 mg of ethyl loflazepate (total concentration of M1 + M2 = 57 ng ml^{-1}, concentration of I.S. = 26 ng). I represents the peak of total M1 + M2, II represents the peak of the internal standard.

caused by emulsion formation. After centrifugation for 10 min at 2000 *g*, the organic phase was transferred to a 3.0-ml mini-vial and then evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50–100 µl of toluene before analysis by GLC.

Determination of unknown samples. The plasma concentration data were obtained from least-square linear regression graphs established daily from four or five calibration points. Peak-height ratios were computed by means of a HP 3388 A system. Quality control samples were analysed together with the unknowns to confirm the assay accuracy; about 10% of the analysed samples were quality controls.

Results and Discussion

Chromatograms

Figure 1 shows typical chromatograms obtained for: (A) control plasma; (B) control plasma spiked with compound M1; (C) plasma from a subject before administration of ethyl loflazepate; and (D) plasma from the same subject 1 h after oral administration of 4 mg of ethyl loflazepate.

Reproducibility

The intra-assay precision (relative standard deviation) and the accuracy (the difference between the determined and expected concentrations) were calculated for plasma concentrations of compound M1 of 0.8 to 550 ng ml^{-1}. The results were acceptable within these concentration ranges (Table 1A).

Likewise, the inter-day precision and accuracy, calculated from results for quality control samples analysed during routine determination of the unknowns, were satisfactory for plasma concentrations of compound M1 of about 26 to 520 ng ml^{-1} (Table 1B).

Table 1
Intra-assay (A) and inter-assay (B) reproducibilities and accuracies for the determination of loflazepate (M1 metabolite) in plasma

	Concentration expected (ng M1 per 0.5 ml)	Concentration determined	*n*	Relative standard deviation (%)	Difference between expected and determined concentrations (%)
A	0.42	0.41	7	6.8	−2.3
	1.70	1.72	6	8.3	+1.7
	3.40	3.67	6	4.8	+7.9
	6.81	7.00	6	3.4	+2.7
	25.90	26.08	7	2.8	+0.7
	64.75	65.08	6	3.1	+0.5
	129.50	130.08	7	1.6	+0.4
	272.50	263.20	7	1.7	−3.4
B	12.88	12.62	6	1.9	−2.0
	25.64	25.49	6	2.5	−0.5
	75.44	74.59	6	2.0	−1.1
	129.17	125.86	6	1.5	−2.6
	257.71	254.51	6	1.6	−1.2

Limit of detection

The limit of detection, (signal-to-noise ratio of about 4 or 5) was approximately 0.2 ng ml^{-1}. Near this detection limit the intra-assay reproducibility and accuracy were better than 10%.

Linearity

Linearity of regression graphs was satisfactory for plasma concentrations of compound M1 of about 3 to 777 ng ml^{-1} plasma; correlation coefficients were 0.9987–0.9998 and the intercepts of the calibration graphs did not differ significantly from zero.

Extraction efficiency

This study was carried out with metabolite M2 dissolved in acetone–hexane (20:80, v/v). Since metabolite M1 is not soluble in organic solvents, it cannot be used as a reference substance in this study. The recovery of compound M2 from human plasma was in the range 71–94%.

Specificity

In respect of possible interference from endogenous plasma components, the specificity of the method was satisfactory (Fig. 1). Interference which might be caused by other compounds, such as benzodiazepines or antiepileptics has not been determined because the method had first to be applied to bioequivalence and bioavailability studies in healthy volunteers who had not received other concurrent medication. However, a feasibility study in those patients with renal disease who had been undergoing polytherapy with drugs such as cyclosporin, azathioprin, frusemide, pristinamycin and amphotericin B, has not shown any interference.

Validation of the assay for various biological fluids

The method has the advantage of being applicable to biological fluids other than plasma. To compare the extraction of M1 from various biological fluids (plasma, whole blood and urine) a Student *t*-test (at 5% significance) was applied to accuracy values (Table 2). Within each group (plasma/whole blood, plasma/urine and whole blood/urine), the difference in observed values was not significant ($t_{exp} < t_{th} = 2.57$), thereby demonstrating that the method is suitable for these biological fluids.

The correlation between the results obtained by the rapid method described in the present work and those obtained by a more complex GLC method [3] was established using about thirty control plasma samples spiked with compound M1. The correlation between the two methods was satisfactory; the equation of the regression curve was $y = 1.0823\ x - 8.0118$ and the correlation coefficient (r) was 0.9952 ($n = 31$) (Fig. 2).

Applications

The method has been applied to plasma and urine samples collected from healthy volunteers after oral administration of one 4-mg tablet or two 2-mg tablets of ethyl loflazepate in bioequivalence trials, and was applied to whole blood, plasma and urine samples after i.v. and i.m. administration of 4 mg of the compound in bioavailability studies. An extensive study of these experimental data will be the subject of a further publication.

Table 2
Validation of the assay for loplazepate (M1) in various biological fluids

	Plasma		Whole blood		Urine	
Concentration expected (ng M1 per 0.5 ml)	Concentration determined (ng M1 per 0.5 ml)	Accuracy (%)	Concentration determined (ng M1 per 0.5 ml)	Accuracy (%)	Concentration determined (ng M1 per 0.5 ml)	Accuracy (%)
1.25	1.15	−8.0	1.26	+0.8	1.16	−7.2
6.25	6.26	+0.1	6.56	+4.9	6.44	+3.0
12.50	12.67	+1.3	13.64	+9.1	12.23	−2.1
62.68	59.75	−4.6	62.01	−1.0	61.20	−2.3
125.37	130.13	+3.7	129.38	+3.2	122.47	−2.3
250.75	250.03	−0.2	254.00	+1.2	259.67	+3.5

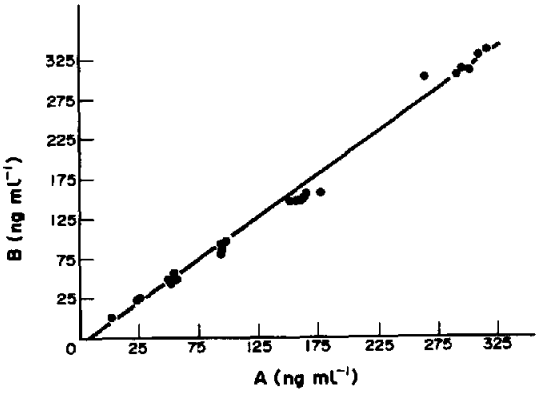

Figure 2
Correlation between the results obtained by (A) the present method and those obtained by (B) a GLC method previously published [3] (concentrations in ng M1 per ml).

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