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DETERMINATION OF CLEBOPRIDE IN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY–NEGATIVE-ION CHEMICAL IONIZATION MASS SPECTROMETRY

PHILIP R. ROBINSON*, MICHAEL D. JONES and JEFFREY MADDOCK

Simbec Research Limited, Merthyr Tydfil, Mid Glamorgan CF48 4DR (U.K.)

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SUMMARY

A procedure for the analysis of clebopride in plasma using capillary gas chromatography–negative-ion chemical ionization mass spectrometry has been developed. Employing an ethoxy analogue as internal standard, the two compounds were extracted from basified plasma using dichloromethane. Subsequent reaction with heptafluorobutyryl imidazole produced volatile monoheptafluorobutyryl derivatives whose ammonia negative-ion mass spectra proved ideal for selected-ion monitoring. The recovery of clebopride from plasma at 0.536 nmol/l was found to be $85.5 \pm 0.9\%$ ($n = 3$) whilst measurement down to 0.268 nmol/l was possible with a coefficient of variation of 7.9%. Plasma levels of the compound are reported in two volunteers following ingestion of 1 mg of clebopride as the malate salt.

INTRODUCTION

Clebopride malate, 4-amino-N-(1-benzyl-4-piperidyl)-5-chloro-*o*-anisamide malate (I, Fig. 1), developed by Laboratorios Almirall, is a potent antidopaminergic benzamide drug possessing antiemetic, gastrokinetic and anxiolytic properties [1–3]. Because of the extensive metabolism of the compound in various species including man [4] and its low dosage (0.5–1 mg) therapeutic plasma levels rarely exceed 2.681–5.362 nmol/l. Consequently, in order to study the disposition and pharmacokinetics of the compound a particularly sensitive and specific technique was required.

Previous attempts to measure clebopride using both thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) [5–7] have been unable to provide the < 1 nmol/l determinations necessary for human studies. Similarly, a method involving acidic decomposition of the compound, fluoroacylation of the liberated 6-chloro-*m*-anisidine and analysis by gas

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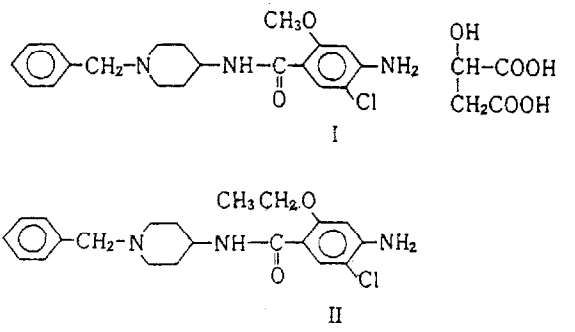


Fig. 1. Structures of clebopride malate (I) and 2-ethoxyclebopride (II).

chromatography–mass spectrometry (GC–MS) had a limit of sensitivity of 5.362 nmol/l [8]. More recently a radioimmunoassay (RIA) technique has been reported to be capable of detecting down to 0.268 nmol/l clebopride in human plasma although the assay reproducibility was only quoted to 1.072 nmol/l [9].

In this paper we describe a sensitive and specific procedure for the analysis of clebopride in human plasma down to 0.268 nmol/l. The method involves production of the mono-heptafluorobutyryl (HFB) derivatives of clebopride and a closely related ethoxy analogue (II, Fig. 1) as internal standard. After separation of the derivatives on a 30-m cross-linked methyl silicone fused-silica capillary column selected-ion monitoring (SIM) of the high-mass negative ions produced by ammonia chemical ionization was then performed. Using the method plasma levels of clebopride have been determined in two volunteers following ingestion of 1 mg of clebopride as the malate salt.

EXPERIMENTAL

Reagents and standards

Methanol, dichloromethane and toluene were HPLC-grade solvents obtained from Rathburn (Walkerburn, U.K.). The toluene was dried over calcium hydride prior to use. Sodium hydroxide was an Analar-grade reagent supplied by BDH (Poole, U.K.). Aqueous solutions were freshly prepared in doubly glass-distilled water. Heptafluorobutyryl imidazole (HFBI) came from Pierce (Cambridge, U.K.) and was stored frozen at −10°C. Clebopride malate and 2-ethoxyclebopride fumarate were supplied by Laboratorios Almirall (Barcelona, Spain). Standard solutions were accurately prepared in volumetric flasks by dissolving an appropriate amount of each salt in methanol to provide a concentration of 0.263 m*M* of free base. Aliquots of the stock solutions were then diluted with methanol to produce working solutions at the required concentration. All solutions were stored in the dark at 4°C.

Preparation of analytical samples and controls

Aliquots (2 ml) of blank, drug-free, pooled plasma were dispensed into disposable 13-ml glass tubes fitted with screw caps (L.I.P., Shipley, U.K.). Each sample was then spiked with a solution of clebopride in methanol in order to provide

concentrations covering the range 0, 0.268, 0.536, 1.340, 2.681 and 6.702 nmol/l. After vortex-mixing for 30 s the tubes were allowed to equilibrate at room temperature for 30 min prior to extraction. A series of blank plasma samples were also extracted along with the foregoing in order to determine the extraction efficiency of the procedure. To the blank extracts were added aliquots of clebopride standard solution to provide amounts equivalent to plasma concentrations of 0.536 and 2.681 nmol/l. These samples were then derivatized and analyzed along with the plasma standards. In addition, randomly numbered, drug-free plasma samples were independently spiked with clebopride and assayed under blind conditions by the analyst.

Clinical study

Following an overnight fast two healthy, male volunteers each received 1 mg of clebopride as the malate salt (two 500-μg Cleboril tablets, Laboratorios Almirall) swallowed with 200 ml of tap water. Blood samples (10 ml) were withdrawn from a forearm vein into lithium heparin Monovettes (Sarstedt, Leicester, U.K.) before, and at the following times after dosing: 0.5, 1, 2, 3, 4, 6, 8, 12, 15 and 24 h. Following gentle mixing the tubes were centrifuged at 1200 *g* (10°C) for 10 min and the plasma component was transferred into a clean tube with the aid of a Pasteur pipette. The tubes were tightly stoppered and the plasma stored frozen at −20°C until just prior to analysis.

Extraction of plasma samples

To 2 ml of plasma in a 13-ml glass tube fitted with a screw cap were added 50 μl of a solution of 2-ethoxyclebopride in methanol (equivalent to 13.02 nmol/l). Following vortex-mixing for 30 s, 1.5 ml of 0.2 *M* sodium hydroxide solution were added, the tubes tightly capped and vortex-mixed for a further 10 s. To each one were then added 7 ml of dichloromethane and the tubes mechanically shaken at 190 cycles/min on a horizontal shaker for 20 min. Centrifugation at 1200 *g* for 10 min was then performed and emulsions at the plasma–dichloromethane interface broken up by stirring with a Pasteur pipette. A further centrifugation then produced a clear lower organic layer from which the upper aqueous phase was removed by aspiration and discarded.

The dichloromethane layer was carefully transferred to a clean tube with the aid of a Pasteur pipette. A stream of nitrogen at room temperature was then used to evaporate each sample to a volume of approximately 1 ml and the tube was vortex-mixed for 1 min. The dichloromethane extract was then transferred to a 2-ml soda glass vial (Kernick, Cardiff, U.K.), using a Pasteur pipette and subsequently evaporated to dryness under a stream of nitrogen. To the residue remaining were added 25 μl of dry toluene and the vial was vortex-mixed for 1 min. Following the addition of 6 μl of HFBI and a further vortex-mix, each sample was placed on a hot-plate at 65°C for 15 min. When cool, the derivatized mixture was again evaporated to dryness under nitrogen. The residue was then reconstituted by the addition of 25 μl of dry toluene and vortex-mixed for 1 min. A 1-μl volume of the resulting solution was injected into the GC–MS system under the conditions described below.

Gas chromatography–mass spectrometry

A Finnigan 4021 quadrupole gas chromatograph–mass spectrometer equipped with an Incos data system, Tektronix 4010 visual display unit and Printronix matrix printer/plotter was used. The data system consisted of a Data General Nova 3 computer containing a Wangco dual disk drive using 5-megabyte magnetic disks.

The mass spectrometer was operated in the chemical ionization mode with a filament current of 350 μA and 80eV electron energy at an ion source temperature of 220 °C. Ammonia was used as reagent gas introduced via a stainless-steel make-up gas line and needle valve to an indicated ionizer forepressure of 50 Pa.

Negative ions at *m/z* 549 and *m/z* 563 were acquired in SIM under computer control using the multiple ion detection (MID) software revision 3.1 of the Incos data system.

The gas chromatograph contained a fused-silica 30 m × 0.25 mm I.D. DB 1 column with a film thickness of 0.25 μm (J and W Scientific, Folsom, CA, U.S.A.). The end of the capillary column was brought through the separator oven and inserted directly into the mass spectrometer transfer line, terminating approximately 1 cm from the electron beam. Helium was used as the carrier gas at a flow-rate of 1.5 ml/min with oxygen and moisture traps installed between the supply cylinder and chromatography column.

Splitless injections were performed using the microprocessor-controlled Grob injection system with the split and septum sweep valves opened 1 min after injection. A 5-μl syringe fitted with a 7.5-cm removable needle (Scientific Glass Engineering, Milton Keynes, U.K.) was used for injection. During analysis the gas chromatograph oven was temperature-programmed from 250 to 300 °C at 20 °C/min. The injection port, separator oven and transfer line temperatures were held isothermally at 280, 290 and 295 °C, respectively.

RESULTS AND DISCUSSION

Characterization of gas chromatographic and mass spectral properties of derivatives

The reaction of clebopride and 2-ethoxyclebopride with HFBI under the conditions described proceeded both rapidly and reproducibly resulting in the formation of mono-HFB derivatives at the primary amine group [10]. The derivatives appeared stable, gave good chromatographic peak shape as shown in Fig. 2 and clebopride-HFB exhibited the electron-impact mass spectrum displayed in Fig. 3. A small molecular ion at *m/z* 569 showed the typical chlorine isotope cluster whilst the most intense fragments occurred at low mass representing ions associated with benzylic and unsaturated piperidyl species [4]. This lack of intense high-mass ions suggested that electron-impact ionization would not produce the necessary sensitivity in SIM of plasma extracts.

Accordingly the mass spectra produced under conditions of ammonia chemical ionization were investigated, the positive-ion mass spectrum of clebopride-HFB being shown in Fig. 4. In contrast to the electron-impact spectrum, low-mass fragments were virtually absent and the majority of the ion current resided in the protonated molecular ion at *m/z* 570. In similar fashion the negative-ion mass

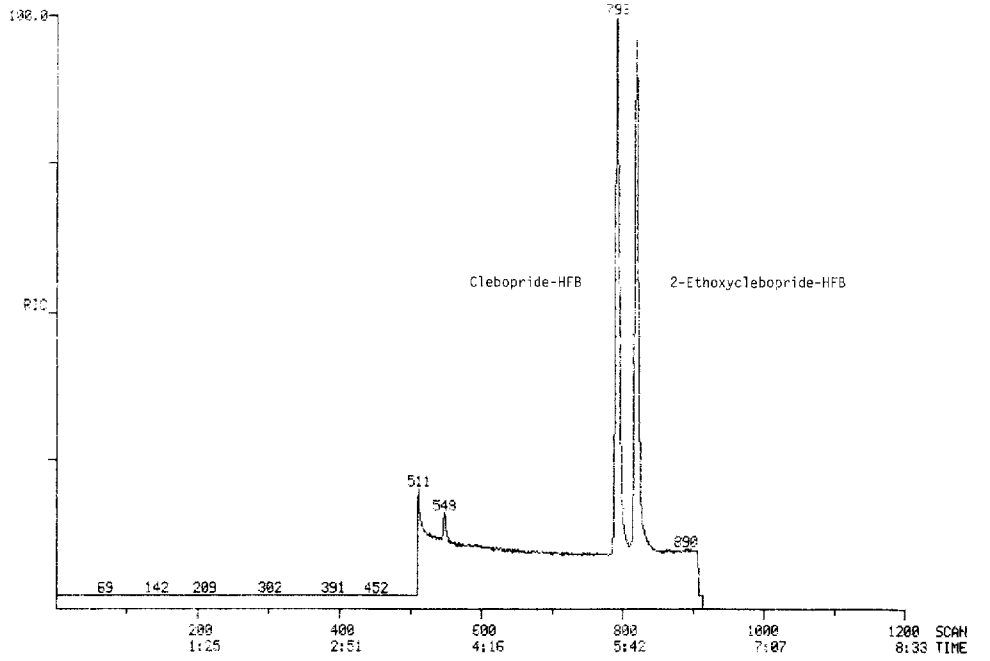


Fig. 2. Separation of clebopride and 2-ethoxyclebopride as their HFB derivatives on a 30-m cross-linked methyl silicone fused-silica capillary column.

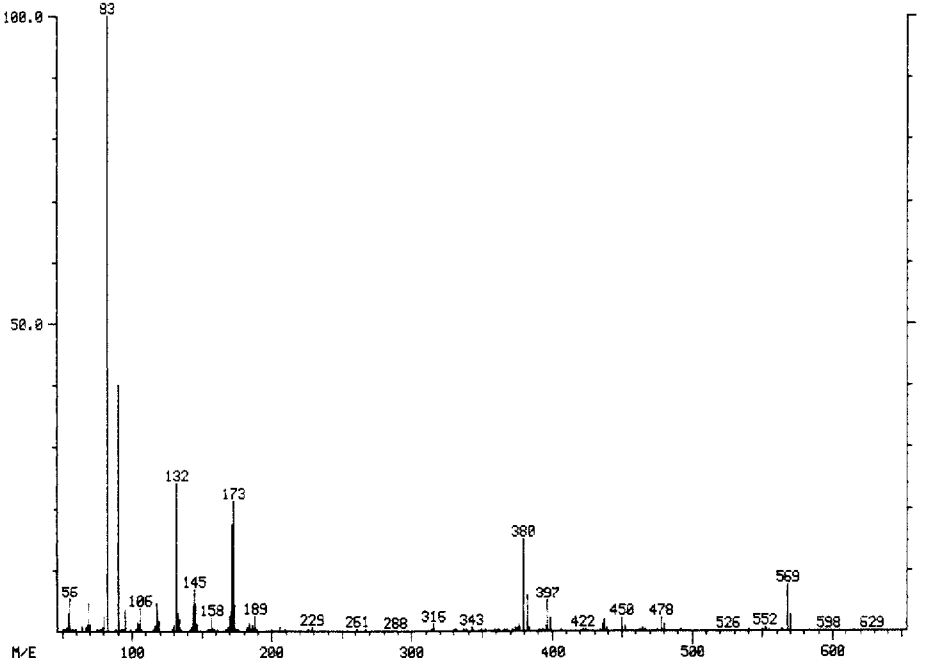


Fig. 3. Electron-impact mass spectrum of the HFB derivative of clebopride.

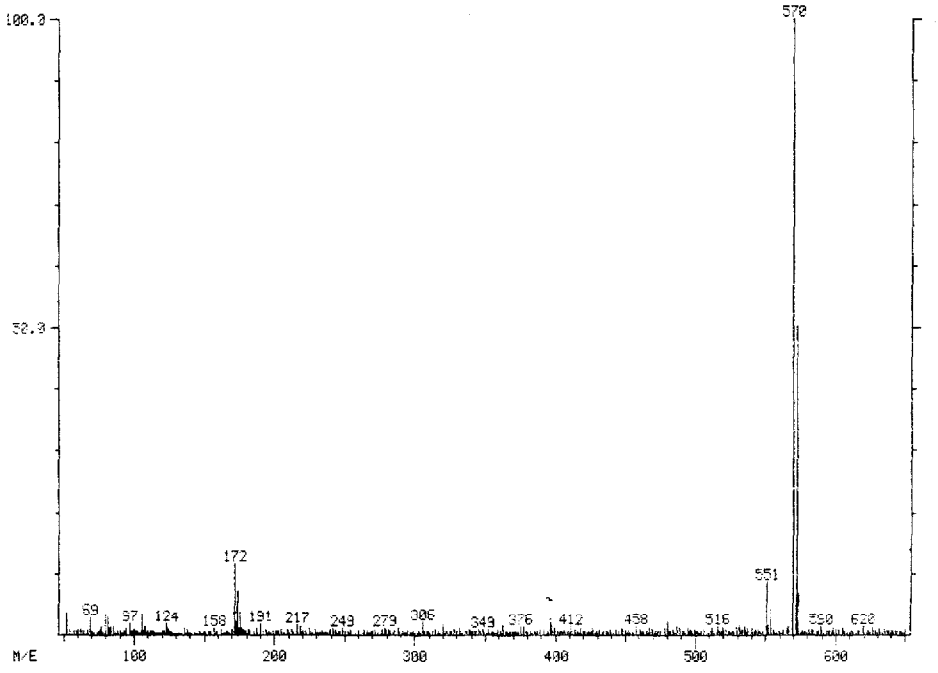


Fig. 4. Positive-ion ammonia chemical ionization mass spectrum of the HFB derivative of clebopride.

spectrum is displayed in Fig. 5A again showing a lack of low-mass fragmentation. However, in the negative-ion mode molecular ion species were virtually absent and replaced by a base peak at *m/z* 549 most probably due to loss of hydrogen fluoride [11]. The corresponding effect was also observed in the negative-ion mass spectrum of the HFB derivative of the internal standard 2-ethoxyclebopride (Fig. 5B), the majority of the ion current residing in the $M^- - 20$ fragment at *m/z* 563.

When monitoring the relative intensities of *m/z* 549 and *m/z* 570 during analysis of equivalent amounts of clebopride-HFB in the negative- and positive-ion chemical ionization modes it appeared that the former gave approximately five times more sensitivity.

Validation of the assay procedure

By SIM of these intense high-mass negative ions in plasma extracts no interference was experienced from co-extracted endogenous material. Measurement of the peak areas of *m/z* 549 and *m/z* 563 at the retention times of the HFB derivatives of clebopride and 2-ethoxyclebopride was performed by the Incos data system. In this way the areas of *m/z* 549 were compared after analysis of the extracted and non-extracted standards (prepared by spiking blank plasma extracts with clebopride). The results showed that the dichloromethane extraction of basified plasma produced recoveries of $85.5 \pm 0.9\%$ ($n=3$) and $86.3 \pm 4.1\%$ ($n=3$) for clebopride at plasma levels of 0.536 and 2.681 nmol/l, respectively. Thus the actual amount of clebopride injected into the GC–MS system from the 0.536 nmol/l sample was 14 pg.

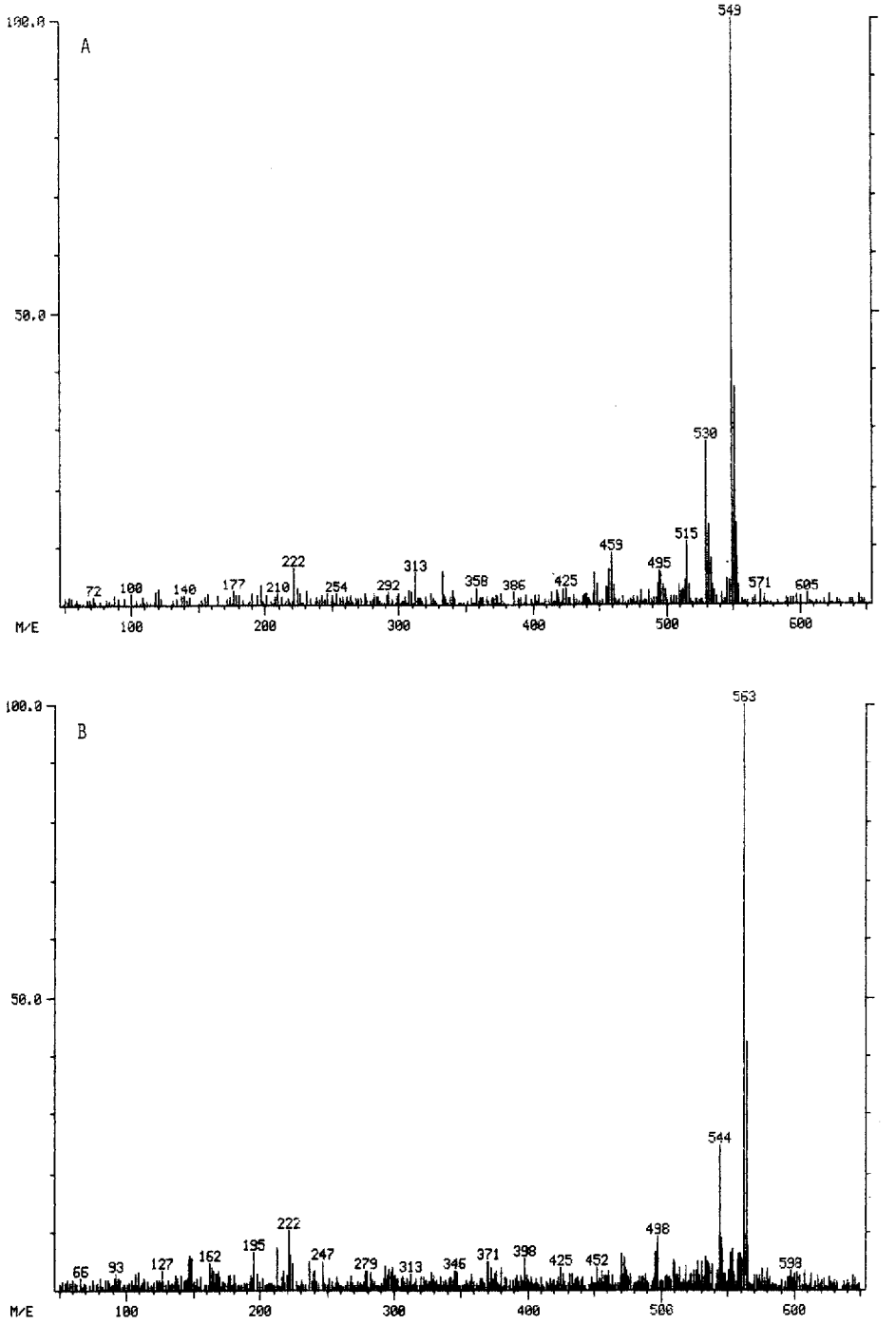


Fig. 5. Negative-ion ammonia chemical ionization mass spectra of the HFB derivatives of (A) clebopride and (B) 2-ethoxyclebopride.

Following analysis of the series of extracted plasma samples the peak-area ratio of clebopride to the internal standard was calculated and plotted against the concentration of drug in the sample. The peak-area ratio information is summarized in Table I along with the standard deviation and coefficient of variation calculated at each concentration. Linear regression parameters covering each calibration line were determined for twelve separate sets of standards analyzed over a period of three months as shown in Table I. The relationship between peak-area ratio and concentration appeared linear over the range 0.268–6.702 nmol/l with a mean correlation coefficient of 0.9999. Good reproducibility was evident over the period of three months with the coefficient of variation ranging from 3.3% ($n=12$) at 6.702 nmol/l to 9.4% ($n=12$) at 0.536 nmol/l.

Results of blind analysis of plasma samples independently spiked with clebopride are presented in Table II and demonstrate a high level of agreement between the actual and calculated values. A number of samples with plasma remaining after analysis were frozen again and placed in storage at $-20\,^{\circ}C$ for five months. After thawing and re-analysis the results obtained did not differ by more than 7% (range -6.7 to $+4.7\%$, $n=6$) from the previous values.

TABLE I

REPRODUCIBILITY OF PEAK-AREA RATIO MEASUREMENTS OVER A PERIOD OF THREE MONTHS

Linear regression analysis of clebopride concentration in plasma (x) versus peak area ratio (y) gave the following results (mean ± S.D.): slope = 9.0981 ± 0.3516; intercept = 0.3169 ± 0.4178; correlation coefficient = 0.9999 ± 0.0003.

Plasma concentration (nmol/l)	Mean peak-area ratio of clebopride to internal standard (%)	n	S.D.	C.V. (%)
0.268	2.908	12	0.231	7.9
0.536	5.444	12	0.512	9.4
1.340	12.253	12	0.479	3.9
2.681	24.949	12	1.116	4.5
6.702	61.221	12	2.047	3.3

TABLE II

RESULTS OF BLIND ANALYSIS OF PLASMA SAMPLES CONTAINING CLEBOPRIDE

Concentration added (nmol/l)	Concentration found (nmol/l)	Percentage difference
0.509	0.509	0
1.314	1.206	−8.2
1.957	1.930	−1.4
2.601	2.627	+1.0
3.351	3.512	+4.8
5.228	5.282	+1.0

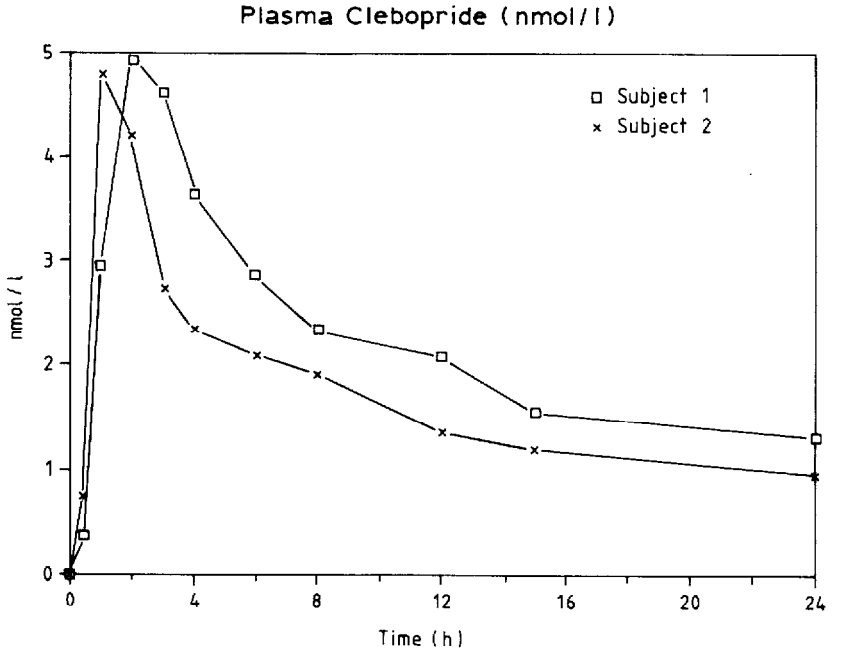


Fig. 6. Plasma concentration versus time profile of clebopride in two male volunteers following oral administration of 1 mg of clebopride as the malate salt.

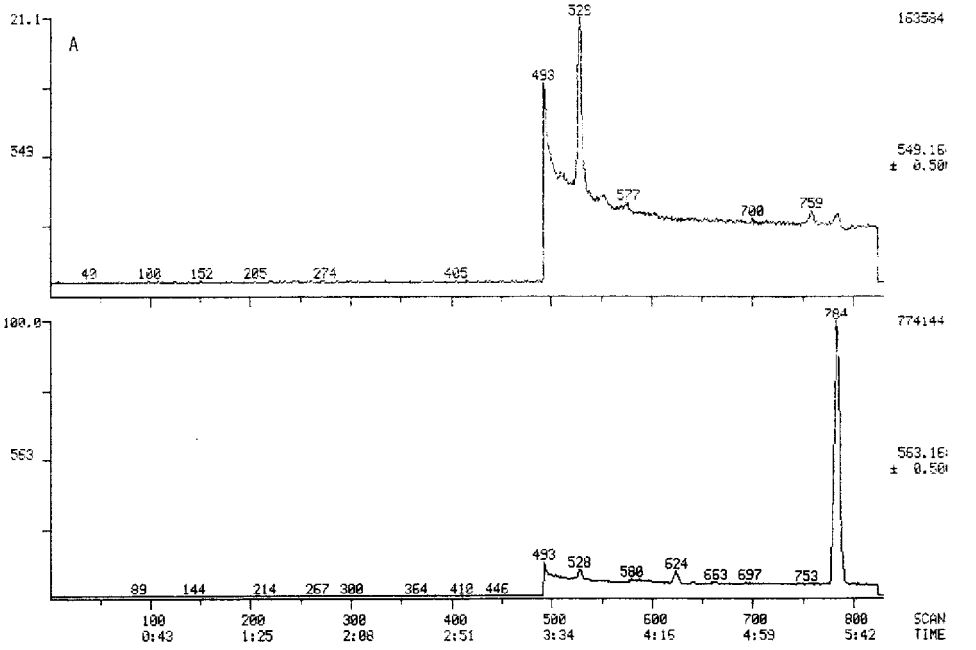
Analysis of clinical study samples

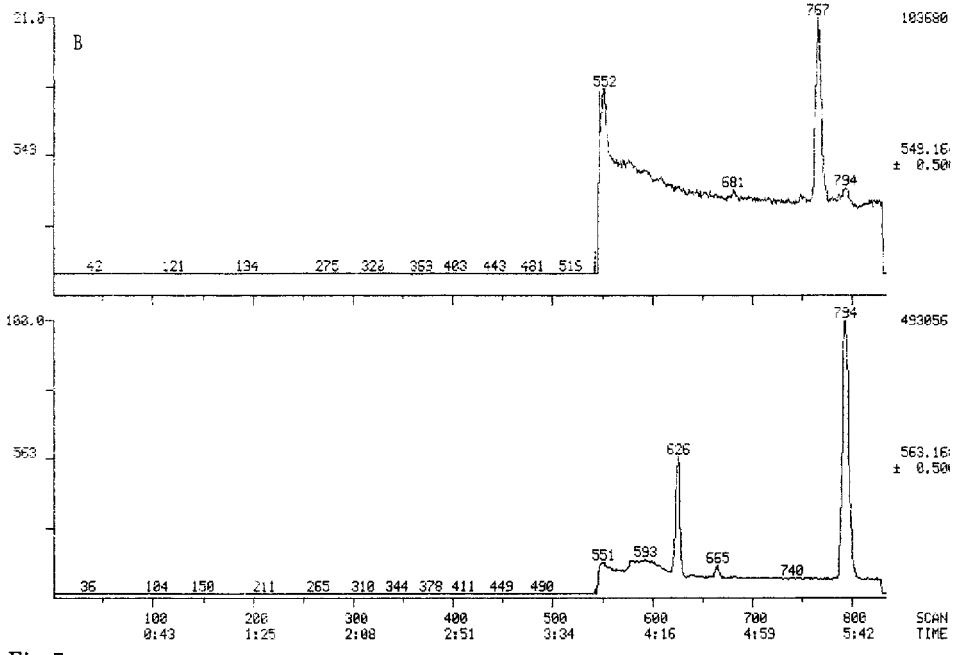
The clinical study was undertaken in order to assess the viability of the methodology when analyzing authentic plasma samples. It would also provide a check for any interference in the assay from clebopride metabolites. The results obtained from the analysis of the clinical study samples are displayed graphically in Fig. 6.

The drug was rapidly absorbed and both subjects showed similar C_{max} values of 4.941 nmol/l (for subject 1 at 2 h after dosing) and 4.823 nmol/l (for subject 2 at 1 h).

The fall-off of the compound in plasma appeared bi-exponential, a rapid decrease in concentration from the C_{max} value being followed by a slower decline beyond 5 h. Appreciable levels were still present in both subjects at 24 h after dosing. Typical SIM traces of *m/z* 549 and *m/z* 563 are shown in Fig. 7 and represent those obtained from a blank plasma sample (Fig. 7A), a clinical study plasma sample containing 0.971 nmol/l (Fig. 7B) and a plasma standard containing 2.681 nmol/l clebopride (Fig. 7C).

It is expected that this new method for the analysis of <1 nmol/l levels of clebopride in plasma will find wider application, especially in the study of the disposition and pharmacokinetics of the drug in human subjects.


A
21.1
549
529
493
577
700
759
42 100 152 205 274 405
163584
549.16
± 0.50
100.0
563
784
493 520 580 624 653 697 753
89 144 214 267 300 364 410 446
774144
563.16
± 0.50
100 0:43
200 1:25
300 2:08
400 2:51
500 3:34
600 4:15
700 4:59
800 5:42
SCAN TIME


B
21.0
549
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SCAN TIME

Fig. 7.

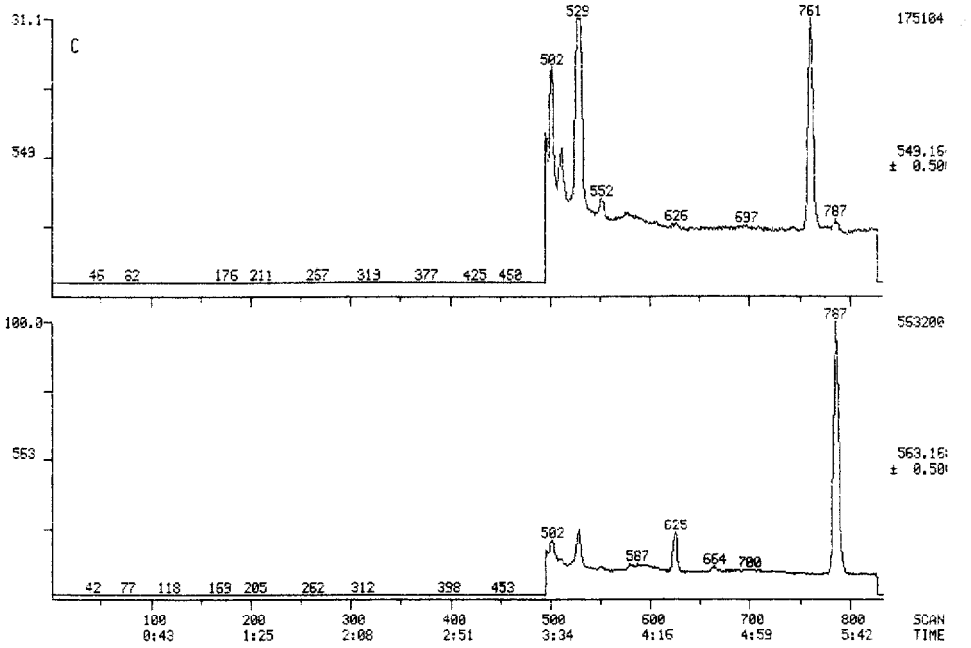

Fig. 7. Typical SIM traces of *m/z* 549 and *m/z* 563 obtained following analysis of (A) a blank plasma sample, (B) a clinical study plasma sample containing 0.971 nmol/l and (C) a plasma standard containing 2.681 nmol/l clebopride.

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