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Simultaneous determination of clebopride and a major metabolite N-desbenzylclebopride in plasma by capillary gas chromatography–negative-ion chemical ionization mass spectrometry

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

Simbec Research Limited, Merthyr Tydfil, Mid Glamorgan CF 48 4DR (U.K.)

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ABSTRACT

A procedure for the simultaneous assay of clebopride and its major metabolite N-desbenzylclebopride in plasma has been developed. The method utilizes capillary gas chromatography–negative-ion chemical ionization mass spectrometry with selected-ion monitoring of characteristic ions. Employing 2-ethoxy analogues as internal standards, the benzamides were extracted from basified plasma using dichloromethane. Subsequent reaction with heptafluorobutyric anhydride produced volatile mono- and diheptafluorobutyryl derivatives of clebopride and N-desbenzylclebopride, respectively. The methane negative-ion mass spectra of these derivatives exhibited intense high-mass ions ideal for specific quantitation of low levels in biological fluids. Using this procedure the recovery of the drug and metabolite from human plasma was found to be 84.4 ± 1.5% (n = 3) and 77.4 ± 4.7% (n = 3), respectively, at 0.5 ng/ml. Measurement of both compounds down to 0.10 ng/ml with a coefficient of variation of less than 10.5% is described. Plasma levels are reported in four volunteers up to 24 h following oral administration of 1 mg of clebopride malate salt.

INTRODUCTION

Clebopride malate, N-(1′-benzyl-4′-piperidyl)-2-methoxy-4-amino-5-chlorobenzamide malate (I, Fig. 1) was initially developed by Laboratorios Almirall as one of a number of substituted benzamide derivatives with promising central antidopaminergic activity. Detailed investigation at several centres has demonstrated its encouraging antiemetic, gastrokinetic and anxiolytic properties [1–3].

The low dosage of the compound required for pharmacological effects in man (0.5–2 mg) and the lack of suitable analytical methodologies had previously precluded the generation of detailed pharmacokinetic information. Early assay attempts using both thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) did not provide the necessary sensitivity below 10 ng/ml [4–6]. However, the development of procedures based on gas chromatography-mass spectrometry (GC–MS) and radioimmunoassay (RIA) has enabled

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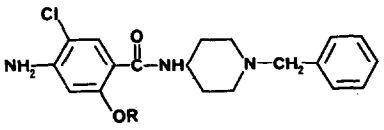
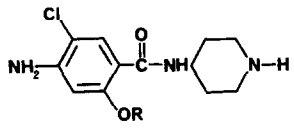


I R=CH_3, as $C_4H_6O_5$ salt III R=C_2H_5



II R=CH_3 IV R=C_2H_5

Fig. 1. Structures of clebopride malate (I), N-desbenzylclebopride (II) and their respective 2-ethoxy internal standards (III and IV).

analysis at low and sub-nanogram per millilitre levels in both animals [7] and man [8,9].

At an early stage in its development clebopride was found to undergo extensive metabolism in a number of animal species including man [10]. As with the related drug metoclopramide [11] the major metabolite was found to be an N-dealkylated product, N-(4′-piperidyl)-2-methoxy-4-amino-5-chlorobenzamide (II, Fig. 1)). None of the previously published methods, however, was capable of measuring this compound and so comparative pharmacokinetic data were lacking.

This present report describes the simultaneous assay of clebopride and its N-dealkylated metabolite down to 0.1 ng/ml in human plasma. Volatile heptafluorobutyryl derivatives of the analytes and their respective internal standards (III and IV, Fig. 1) are prepared prior to analysis by negative-ion chemical ionization (NICI) GC–MS. Using this method plasma levels of both compounds have been determined in four subjects up to 24 h following ingestion of 1 mg of clebopride as the malate salt.

EXPERIMENTAL

Materials

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 double glass-distilled water. Heptafluorobutyric anhydride (HFBA) came from Pierce (Chester, U.K.) and was stored frozen at −10°C. Clebopride malate, 2-ethoxyclebopride, N-desbenzylclebopride and 2-ethoxy-N-desbenzylclebopride were supplied by Lab-

oratorios Almirall (Barcelona, Spain). Standard solutions were accurately prepared in volumetric flasks by dissolving an appropriate amount of each compound in methanol to provide a concentration of 1000 ng/ml 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 standards 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 solutions of clebopride and N-desbenzylclebopride in methanol in order to provide concentrations covering the range 0, 0.10, 0.25, 1.02, 4.06 and 16.24 ng/ml of the drug and 0, 0.10, 0.27, 1.08, 4.33 and 17.34 ng/ml of the metabolite. After vortex-mixing for 30 s the tubes were allowed to equilibrate at room temperature for 30 min prior to addition of internal standards and extraction as described in the following section. A series of blank plasma samples were also extracted along with the foregoing in order to determine the extraction efficiency of the procedure. To these blank extracts were added aliquots of the standard solutions to provide amounts equivalent to plasma concentrations of 0.50 and 5.00 ng/ml. These samples were then derivatized and analyzed along with the plasma standards. In addition, randomly numbered, drug-free plasma samples were independently spiked with both compounds and assayed under blind conditions by the analyst.

Extraction of plasma samples

To 2 ml of plasma (standard, control or clinical study sample) in a 13-ml glass tube fitted with a screw cap were added 50 μl of a solution containing 2-ethoxyclebopride and 2-ethoxy-N-desbenzylclebopride in methanol (equivalent to 10 and 20 ng/ml, respectively). 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 were 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 50 μl of dry toluene and the vial was vortex-mixed for 1 min. Following the addition of 5 μl of heptafluorobutyric anhydride 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 50 μl of dry toluene and vortex-mixed for 1 min. A 1-μl aliquot of the resulting solution was injected into the GC–MS system under the conditions described below.

Gas chromatography-mass spectrometry

A Hewlett Packard (HP 5988) quadrupole mass spectrometer was used during this investigation coupled via a capillary direct interface to an HP 5890 gas chromatograph. The data system comprised an HP 1000 RTE-A Series Micro 14 computer using an 81-Mbyte Winchester disc drive, Thinkjet printer and HP 2397A colour graphics terminal.

Electron-impact (EI) mass spectra were obtained at 70 eV electron energy with a filament current of 300 μA. In the chemical ionization mode a filament current of 380 μA and electron energy of 200 eV were used with methane as reagent gas introduced via a stainless-steel make-up gas line and needle valve to an ion source pressure of 1.8 Torr and temperature of 260°C.

Mass calibration and tuning was effected using perfluorotributylamine and the pre-programmed data system functions Autotune and Manual Tune. Negative ions at m/z 549, 563, 655 and 669 were acquired in selected-ion monitoring (SIM) using dwell times of 100 ms per ion.

The gas chromatograph contained a fused-silica 15 m × 0.25 mm I.D. DB 5 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 inserted directly into the mass spectrometer ion source, 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 into the glass-lined capillary injection port using the HP 7673A autoinjector. This was operated in the batch analysis mode using a robotic arm autosampler controlled via the HP 1000 computer. Split (50 ml/min) and septum sweep (2 ml/min) valves were opened automatically 1 min after injection. During analysis the gas chromatograph oven was temperature-programmed from 130 to 300°C at 30°C/min. The injection port and interface temperatures were held isothermally at 270 and 300°C, respectively.

RESULTS AND DISCUSSION

Characterization of gas chromatographic and mass spectral properties of derivatives

Previous work in this laboratory [9] had demonstrated that the reaction of clebopride and 2-ethoxyclebopride with heptafluorobutyryl imidazole (HFBI) proceeded rapidly and reproducibly at the primary amine group with the produc-

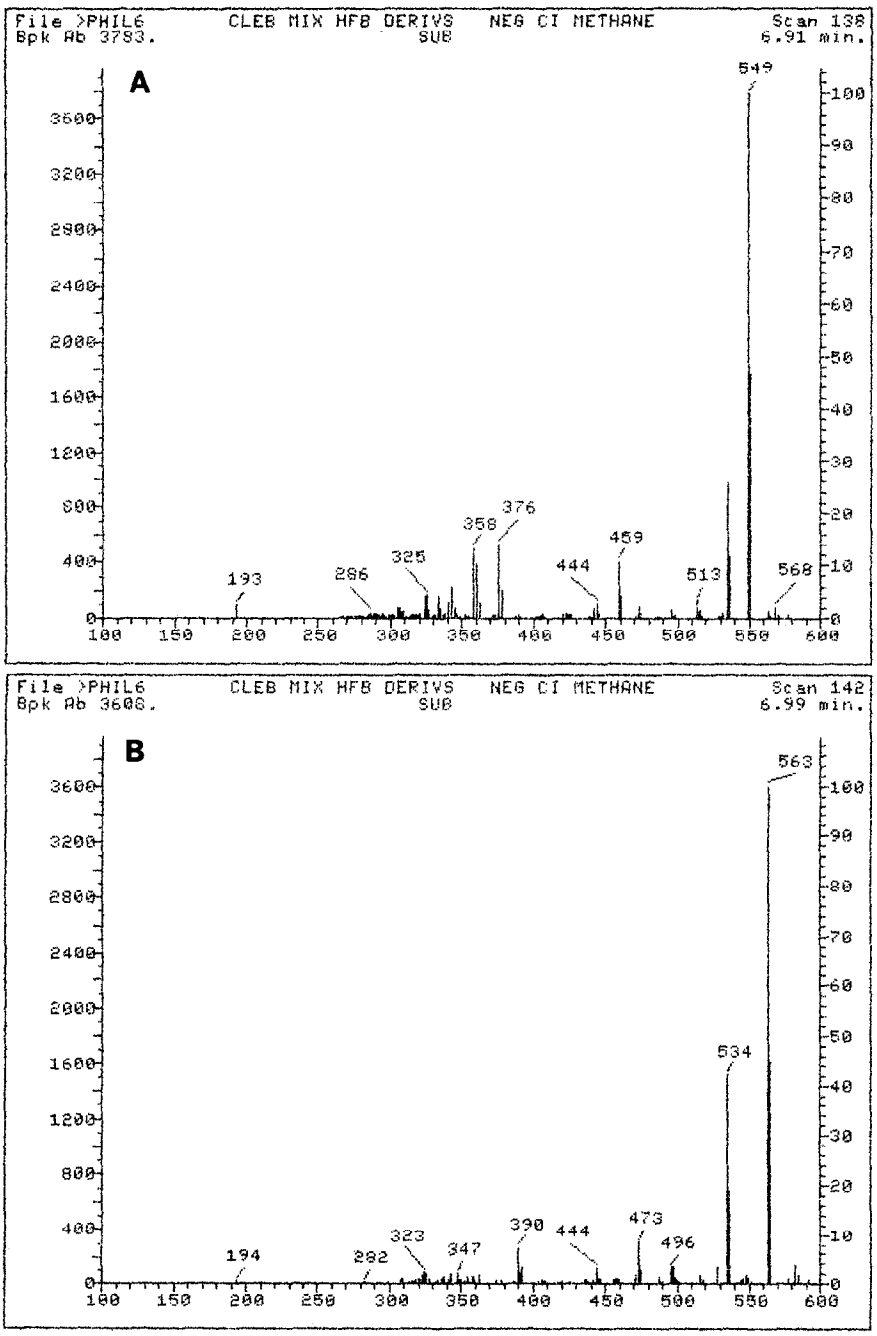


Fig. 2. Negative-ion methane chemical ionization mass spectra of the mono-HFB derivatives of (A) clebopride and (B) 2-ethoxyclebopride.

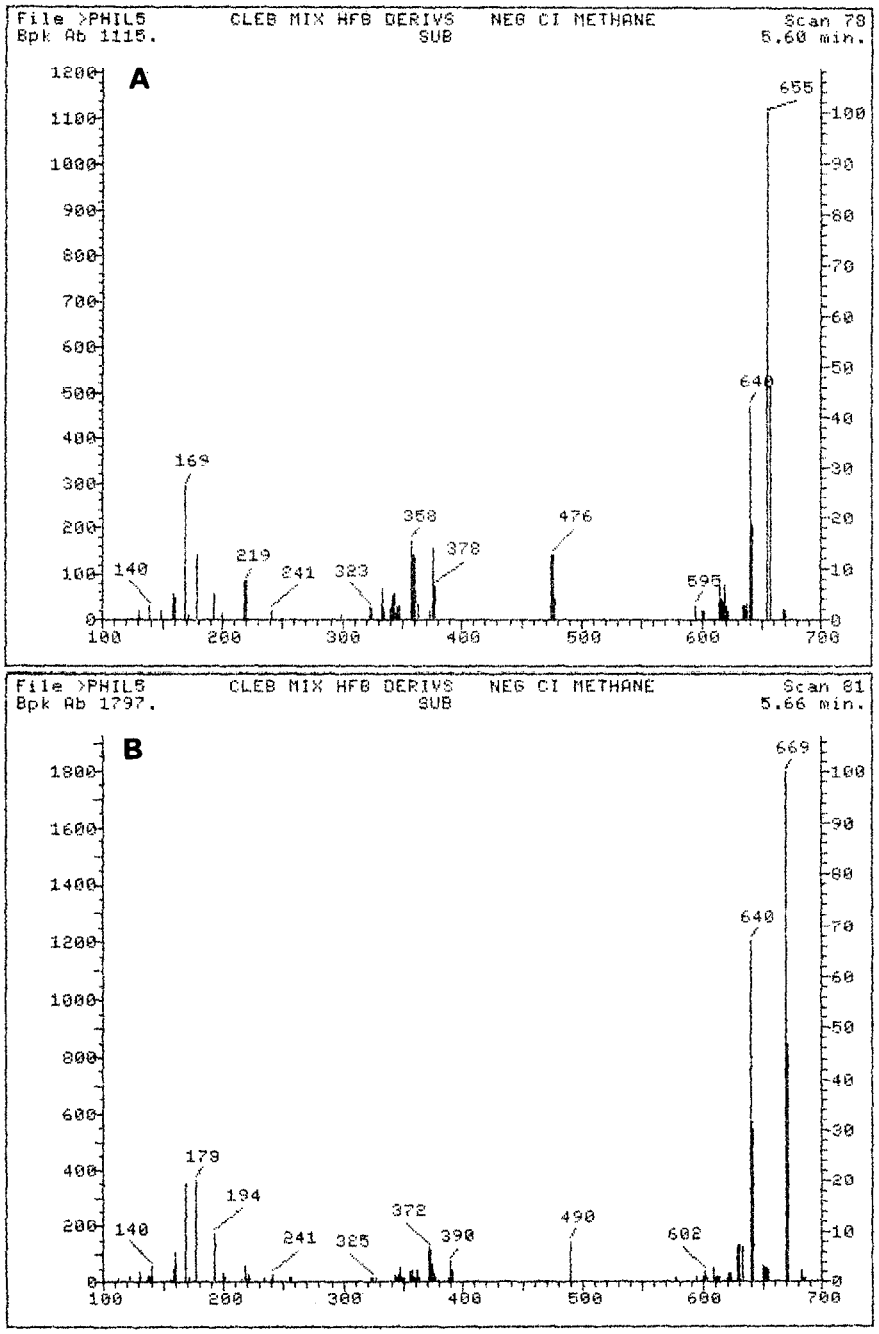


Fig. 3. Negative-ion methane chemical ionization mass spectra of the di-HFB derivatives of (A) N-desbenzylclebopride and (B) 2-ethoxy-N-desbenzylclebopride.

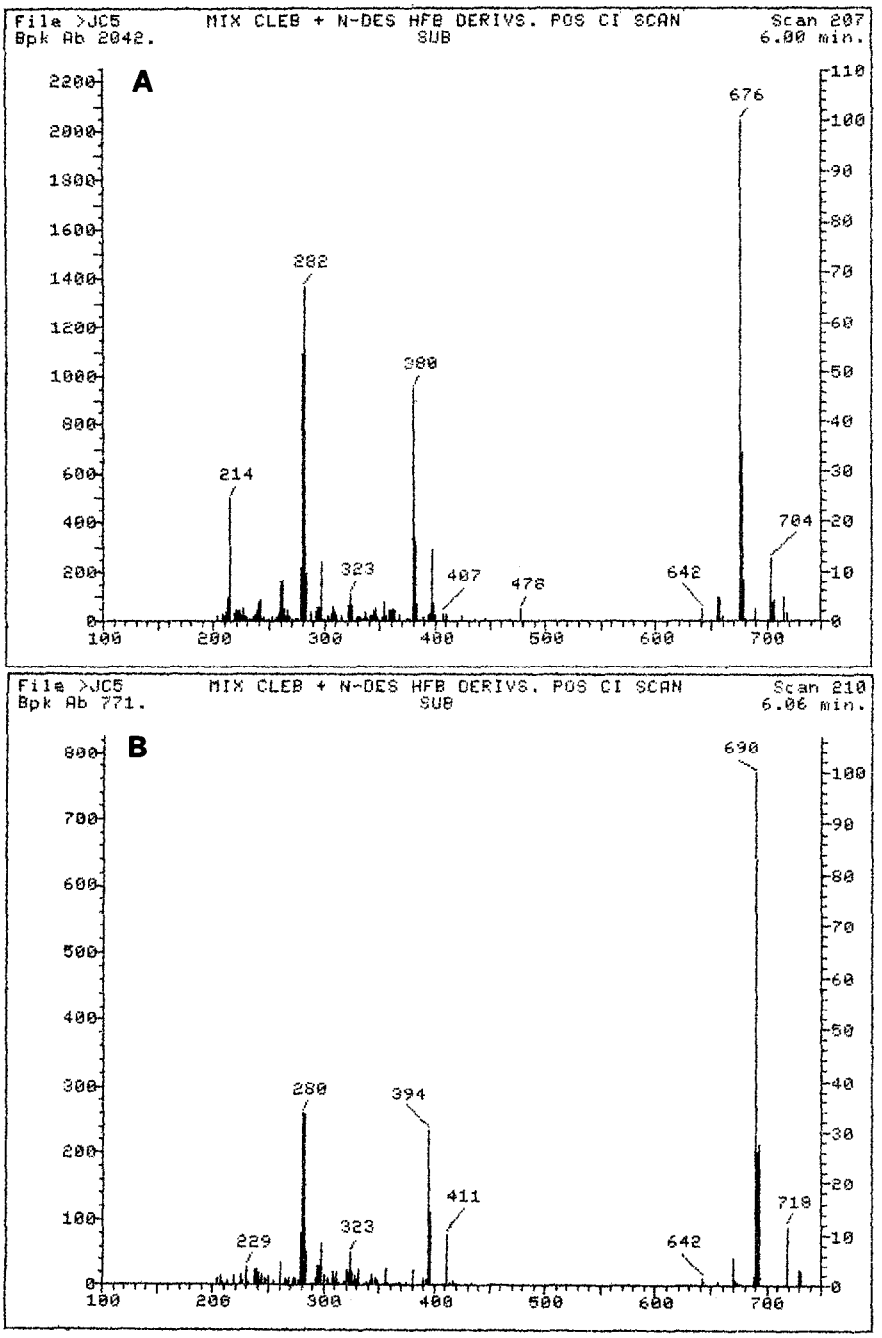


Fig. 4. Positive-ion methane chemical ionization mass spectra of the di-HFB derivatives of (A) N-desbenzylclebopride and (B) 2-ethoxy-N-desbenzylclebopride.

tion of stable mono-HFB derivatives [12]. It was subsequently found that the corresponding anhydride, HFBA, reacted equally well under similar conditions, whilst the removal of excess reagent was easily achieved by evaporation. The resulting background-subtracted negative-ion mass spectra of the derivatives are displayed in Fig. 2. Molecular ion species were virtually absent and the base peaks corresponded to facile loss of hydrogen fluoride [13] giving m/z 549 and 563 for clebopride and internal standard derivatives, respectively.

In similar fashion, N-desbenzylclebopride and 2-ethoxy-N-desbenzylclebopride were found to give the expected products following fluoroacylation, the negative-ion mass spectra of which are shown in Fig. 3. On this occasion, di-HFB derivatives were obtained, reaction having occurred at the piperidine N–H group as well as the primary amine. As with the mono-HFB derivatives of the drug and internal standard, loss of hydrogen fluoride produced the base peaks in the negative-ion spectra giving intense m/z 655 and 669 fragments for metabolite and internal standard, respectively. Confirmation of this assignment was provided by an examination of the corresponding positive-ion chemical ionization mass spectra depicted in Fig. 4. The acylated products possessed protonated molecular ions at m/z 676 and 690 as base peaks indicating the presence of di-HFB derivatives of metabolite and internal standard.

Thus, the majority of the ion current produced on NICI of these compounds resided in specific intense high-mass fragments eminently suitable for SIM. Production of the di-HFB derivatives appeared to proceed as efficiently as a single substitution at the primary amine group. Reaction with HFBA for longer than 15 min did not result in an increase in SIM peak area for the metabolite and internal standard derivatives and the ratio of these to the corresponding mono-substituted products remained constant. Fig. 5 shows the excellent capillary GC peak shape and separation of the derivatives obtained along with the associated mass chromatograms of their negative-ion base peaks. Despite the metabolite and internal standard derivatives having molecular weights over 100 mass units greater than the other pair, their retention times were 1.2 min shorter at 5.6 min owing to the extra volatility conferred by the additonal HFB group. The seven extra fluorine atoms also provided enhanced negative-ion sensitivity, with the earlier-eluting peaks having approximately twice the intensity of the later pair.

Validation of the assay procedure

In order to determine the viability of the method for simultaneous analysis of both clebopride and its major metabolite in plasma samples, SIM of m/z 549, 563, 655 and 669 was performed in several series of spiked samples. Use of these specific high masses ensured no interference from endogenous plasma components and measurement of peak areas at the retention times of the mono- and di-HFB derivatives by the HP 1000 computer accurately reflected the concentration of each compound in the plasma matrix.

Accordingly, the areas of m/z 549 and 655 were measured in plasma samples

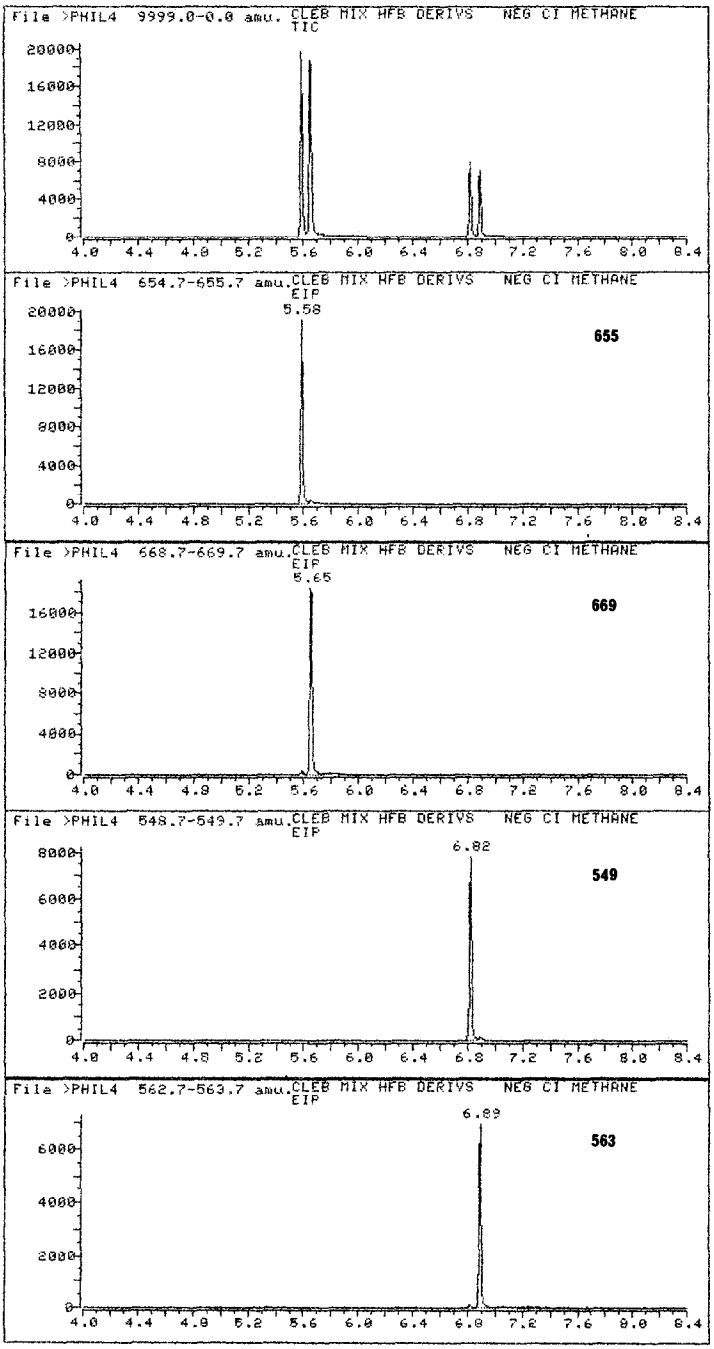


Fig. 5. Total-ion current and mass chromatograms of NICI base peaks obtained following derivatization of a standard mixture of all analytes with HFBA.

TABLE I

EXTRACTION EFFICIENCY OF CLEBOPRIDE AND N-DESBENZYLCLEBOPRIDE FROM HUMAN PLASMA

$n = 3$.

Sample	Concentration (ng/ml)	Mean SIM peak area	(Extracted/non-extracted) × 100 (mean ± S.D.)
Clebopride derivative, m/z 549			
Plasma extract	0.5	153 305	84-5 ± 1.5
Non-extracted	0.5	181 509	
Plasma extract	5.0	2 651 850	83.9 ± 3.9
Non-extracted	5.0	3 162 453	
N-Desbenzylclebopride derivative, m/z 655			
Plasma extract	0.5	184 750	77.3 ± 4.7
Non-extracted	0.5	238 959	
Plasma extract	5.0	2 245 477	72.0 ± 3.4
Non-extracted	5.0	3 117 107	

TABLE II

REPRODUCIBILITY OF SIM PEAK-AREA RATIO MEASUREMENTS OVER A PERIOD OF SIX WEEKS

$n = 30$.

Plasma concentration (ng/ml)	Peak-area ratio analyte/internal standard (mean ± S.D.) (%)	Coefficient of variation (%)
Clebopride		
0.10	0.84 ± 0.08	9.5
0.25	2.31 ± 0.19	8.2
1.02	9.48 ± 0.56	5.9
4.06	41.19 ± 2.28	5.5
16.24	165.04 ± 8.04	4.9
N-Desbenzylclebopride		
0.10	0.69 ± 0.07	10.1
0.27	1.41 ± 0.13	9.2
1.08	5.08 ± 0.29	5.7
4.33	20.29 ± 1.31	6.5
17.34	83.22 ± 4.88	5.9

Unweighted linear regression analysis of analyte concentration in plasma (x) *versus* peak-area ratio (y) gave the following results (mean ± S.D.):

	Clebopride	N-Desbenzylclebopride
Slope	10.1978 ± 0.5311	4.7103 ± 0.3181
Intercept	−0.4187 ± 0.3964	0.0920 ± 0.1229
Correlation coefficient	0.9995 ± 0.0005	0.9985 ± 0.0014

originally spiked in triplicate with both compounds at 0.50 and 5.00 ng/ml. These results were then compared to those obtained after analysis of the non-extracted standards prepared by spiking blank plasma extracts with equivalent amounts of clebopride and N-desbenzylclebopride. The data are presented in Table I and demonstrated the high (>70%) and reproducible extraction efficiency for both compounds into dichloromethane from basified plasma. Removal of the benzyl group had obviously increased the hydrophilic nature of the metabolite with a consequent lowering of its recovery into the organic solvent. The actual amount of each material injected into the GC–MS system following extraction and derivatization from a 0.10 ng/ml plasma sample was less than 4 pg.

Over a period of six weeks thirty sets of blank plasma samples were spiked with clebopride and N-desbenzylclebopride, extracted, derivatized and analyzed according to the procedure described herein. The SIM peak areas were measured and the area ratios of both analytes to their respective internal standards were calculated and plotted against the concentration of each compound in the sample over the range 0.10–17.34 ng/ml. This peak-area ratio information is summarized in Table II along with the standard deviation and coefficient of variation calculated at each concentration. Unweighted linear regression parameters covering individual calibration lines were determined for the thirty sets of drug and metabolite standards analyzed throughout the investigation. For both compounds the relationship between peak-area ratio and concentration appeared linear over the range 0.10–17.34 ng/ml, mean correlation coefficients exceeded 0.9980 (Table II). Good assay reproducibility was also evident over the period of six weeks, even at the lowest level of quantitation (0.10 ng/ml) the coefficient of variation did not exceed 10.1% for either compound (clebopride 9.5%, N-desbenzyl clebopride 10.1%, $n = 30$).

As a further check on the overall validity of the methodology, independently spiked, randomly numbered plasma samples were routinely assayed on a blind

TABLE III

RESULTS OF BLIND ANALYSIS OF PLASMA SAMPLES CONTAINING CLEBOPRIDE AND N-DESBENZYLCLEBOPRIDE

Analyte	Concentration added (ng/ml)	Concentration found (mean ± S.D., $n = 12$) (ng/ml)	Percentage difference	Coefficient of variation (%)
Clebopride	0.30	0.32 ± 0.02	+6.7	6.3
	1.59	1.51 ± 0.08	−5.0	5.3
	11.90	11.25 ± 0.35	−5.5	3.1
N-Desbenzyl-clebopride	0.32	0.30 ± 0.02	−6.3	6.7
	1.70	1.71 ± 0.08	+0.6	4.7
	12.72	12.37 ± 0.53	−2.8	4.3

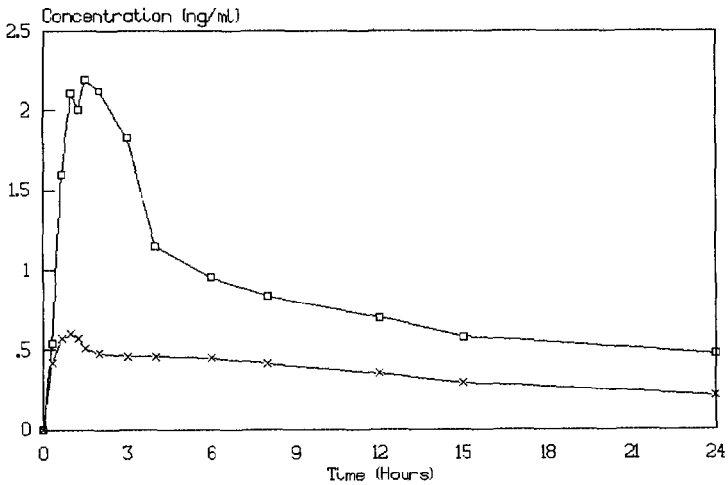


Fig. 6. Mean plasma concentration *versus* time profile of clebopride (□) and N-desbenzylclebopride (×) in four healthy male volunteers following oral administration of 1 mg of clebopride as the malate salt.

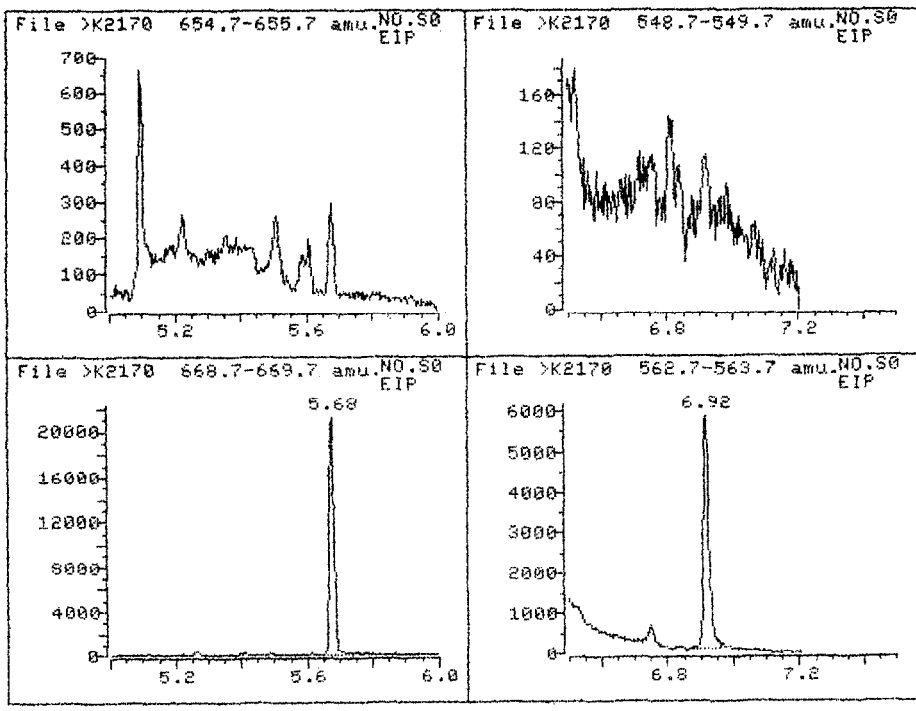


Fig. 7. Typical SIM trace obtained at *m/z* 549, 563, 655 and 669 following analysis of a blank plasma sample.

basis along with the standards. The levels of clebopride and N-desbenzylclebopride were calculated using the appropiate calibration line and the results are presented in Table III. Close agreement was obtained between measured and actual values for both compounds throughout the calibration range. This suggested that the method could be used with confidence for assay of samples from pharmacokinetic studies in man. Because of the large numbers of samples generated in such studies and the need for clinical and analytical phases often to be performed at different sites it was inevitable that storage of samples would sometimes be necessary prior to analysis. Results from this laboratory had previously demonstrated that plasma samples containing clebopride could be stored at −20°C for at least five months with no evidence of analyte decomposition [9]. In similar fashion a number of samples containing known amounts of N-desbenzylclebopride were frozen and placed in storage for six months. On thawing and re-assay the results obtained did not differ by more than 9.5% (range −9.3% to +4.1%, $n = 6$) from the previous values.

Monitoring of drug and metabolite levels in clinical study samples

Blood samples were collected by venepuncture from four healthy male volunteers at various times following oral administration of 1 mg of clebopride as the

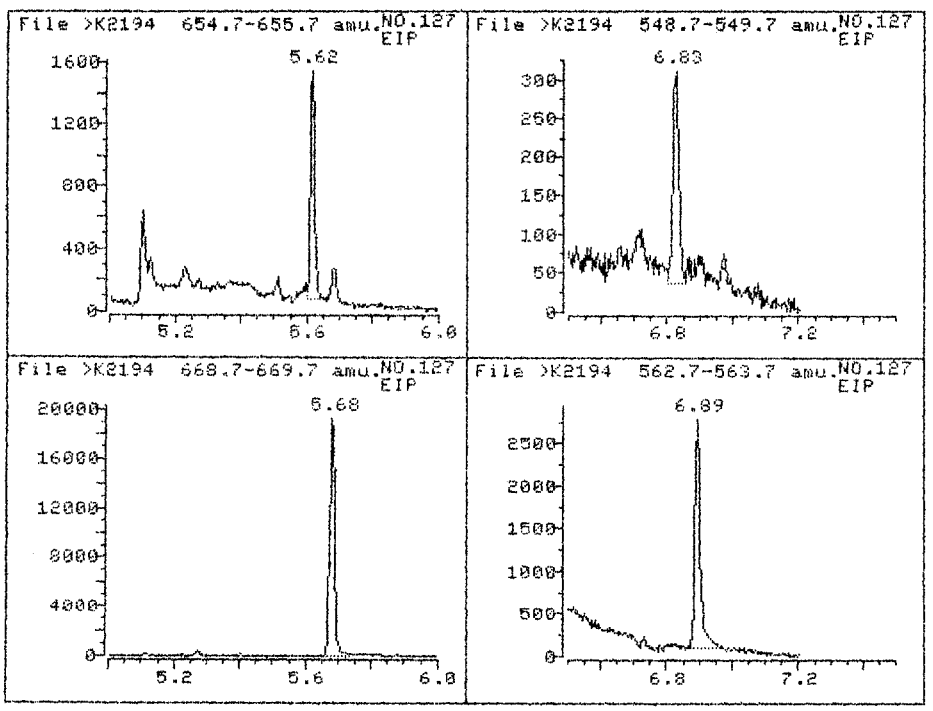


Fig. 8. Typical SIM trace obtained at *m/z* 549, 563, 655 and 669 following analysis of a clinical study sample containing 0.95 ng/ml clebopride and 0.48 ng/ml N-desbenzylclebopride.

malate salt. After centrifugation the plasma component was separated and transferred into clean polystyrene tubes prior to storage at −20°C until just before analysis. Samples were obtained from each subject prior to dosing and at 0.33, 0.67, 1, 1.25, 1.5, 2, 3, 4, 6, 8, 12, 15 and 24 h following drug administration. Aliquots (2 ml) of plasma were taken for assay along with spiked standard and control samples prepared as described previously.

The results obtained are displayed graphically in Fig. 6 with the mean of the individual clebopride and N-desbenzylclebopride plasma levels provided up to 24 h after dosing. Although the drug was swiftly absorbed, the mean C_{max} at 1.5 h was only 2.19 ± 0.42 ng/ml. N-Desbenzylation of clebopride appeared to occur very rapidly the T_{max} being even earlier than that for the unchanged drug. Plasma levels of the metabolite (C_{max} 0.60 ± 0.26 ng/ml) ranged from 0.23 to 0.78 × those of clebopride. Clebopride plasma concentrations appeared to decline exponentially whilst the metabolite was eliminated more slowly with a longer half-life and appreciable levels of both compounds (>0.20 ng/ml) were still present after 24 h.

Figs. 7–9 display typical SIM traces obtained at *m/z* 549, 563, 655 and 669 following analysis of a blank plasma sample (Fig. 7), a clinical study sample

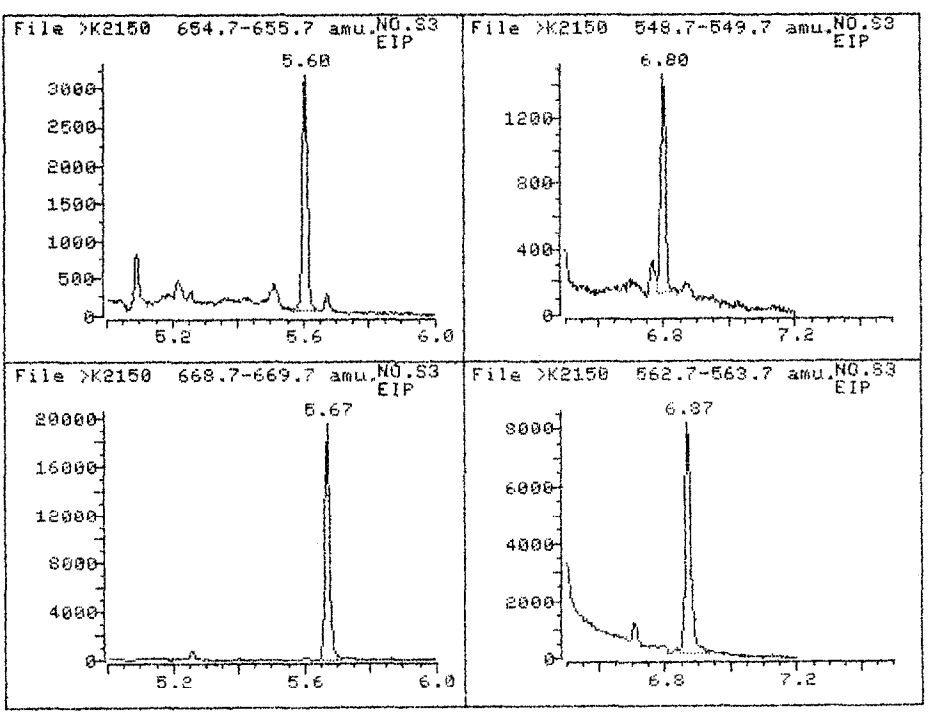


Fig. 9. Typical SIM trace obtained at *m/z* 549, 563, 655 and 669 following analysis of a plasma standard containing 1.00 ng/ml clebopride and N-desbenzylclebopride.

containing 0.95 and 0.48 ng/ml of drug and metabolite, respectively (Fig. 8) and a plasma standard containing 1.0 ng/ml of both compounds (Fig. 9). The peaks due to the derivatized analytes and internal standards were clearly visible with no interference from co-eluting endogenous plasma components.

Despite the fact that at therapeutic doses of clebopride (0.5–2 mg) plasma levels rarely exceed 5 ng/ml, the present method is able to provide valuable pharmacokinetic information for both the unchanged drug and its N-desbenzyl metabolite. At present the assay has been used for several thousand samples from various human and animal studies and has thus demonstrated its robustness. The method has also been shown to be sensitive enough to assay both compounds in samples of human saliva.

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