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SENSITIVE ELECTRON-CAPTURE GAS–LIQUID CHROMATOGRAPHIC ASSAY FOR THE DE-ETHYLATED METABOLITE OF METOCLOPRAMIDE

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SUMMARY

A procedure is described for determining 4-amino-5-chloro-2-methoxy-N-(2-ethylaminoethyl)benzamide, a metabolite formed by de-ethylation of metoclopramide, in urine from rats. The sample is extracted (at pH $\approx$ 13) with chloroform and, after treatment with heptafluorobutyric anhydride, derivatives of the extracted compounds are analyzed by gas–liquid chromatography, with electron-capture detection and diazepam as internal standard. The drug and its metabolite are separated, and the latter can be determined in the range 0.4 to 1.85 μg/ml in the sample. The behaviour of the metabolite during chemical-ionization and electron-impact mass spectrometry is discussed.

INTRODUCTION

Little is known about the fate of metoclopramide (MCP) in animals and man. The inability to measure trace levels has been the principal reason for the lack of comprehensive studies of this drug and its metabolites. A sensitive and specific analytical method is therefore required to detect the parent drug and its major metabolites if study of the compound is to proceed. A highly sensitive electron-capture gas–liquid chromatographic (GLC–ECD) assay for MCP has recently been reported for use in rat-plasma studies[1]; no major peaks indicating the presence of metabolites were observed on the chromatograms obtained from the samples[1].

N-De-ethylation has been shown to be the major metabolic reaction in rat[2], and the de-ethylated metabolite (DE-MCP), 4-amino-5-chloro-2-methoxy-N-(2-ethylaminoethyl)benzamide, has been reported to have a similar, but less potent, pharmacological action[3]. This metabolite has also been identified in the current cumulative urine studies. By using a technique similar to that described in the previous paper[1], a sensitive GLC–ECD assay has been devised to quantitate the metabolite in rat urine.

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EXPERIMENTAL

Materials

4-Amino-5-chloro-2-methoxy-N-(2-ethylaminoethyl)benzamide was supplied by A. H. Robins Co. (Richmond, Va., U.S.A.). Heptafluorobutyric anhydride (HFBA) was purchased from Pierce (Rockford, Ill., U.S.A.). Diazepam was supplied by Hoffmann-La Roche (Montreal, Canada). 1 *N* sodium hydroxide and 4% ammonium hydroxide were prepared from BDH and ACS reagent grade, respectively. Chloroform (distilled in glass) was obtained from Caledon (Georgetown, Ontario, Canada).

GLC

A Hewlett-Packard gas chromatograph (model 5840) equipped with a ^{63}Ni ECD and a glass column (1.2 m × 2 mm I.D.) containing 3% of SP-2250 DB coated on Supelcoport (100–120 mesh) was used. The operating conditions for routine analysis were: injection temperature, 250°; oven temperature 235°; detector temperature, 350°; carrier gas [argon–methane (19:1, v/v)] flow-rate, 40 ml/min.

Extraction and derivative formation

To 1 ml of blank urine containing DE-MCP was added 1 ml of 1 *N* NaOH (pH ≈ 13) and 6 ml of chloroform, and the mixture was shaken on a horizontal shaker for 20 min to extract the DE-MCP. After centrifugation, 5 ml of the organic phase was transferred to a 15-ml centrifuge tube, and the contents were dried under a gentle stream of nitrogen. The residue was reconstituted with 1 ml of internal-standard solution (1 μg/ml of diazepam in benzene) and 20 μl of heptafluorobutyric anhydride (HFBA) were added. After thorough mixing (vortex-type mixer), the reaction mixture was incubated at 55° for 20 min, then allowed to cool, and the excess of derivatizing agent was removed by hydrolysis with 0.5 ml of water and neutralization with 0.5 ml of 4% ammonium hydroxide solution.

Mass spectrometry (MS)

Electron impact (EI). A Varian Mat-111 GLC–electron impact (EI) mass spectrometer was used to study the heptafluorobutyryl (HFB) derivative of DE-MCP. The following conditions were used: for GLC, the injection and oven temperatures were 250° and 230°, respectively, the carrier-gas (helium) flow-rate was 20 ml/min. and a 1.8-m. × 2-mm I.D. glass column packed with 3% of OV-17 coated on Chromosorb W (80–100 mesh) was used. For MS, the ionization energy was 70 eV, the electron-multiplier voltage was 2 kV, the analyzer temperature was 50° and the separator-oven temperature was 200°.

Chemical ionization (CI). A Finnigan GLC–CI mass spectrometer (Model 4000) was employed to identify the molecular ion of the HFB derivative. The GLC conditions used were as described for routine analysis except that the glass column (0.6 m × 2 mm I.D.) contained 3% of OV-101 coated on Chromosorb W (80–100 mesh), and methane was used as a carrier and reagent gas (flow-rate 40 ml/min). The separator oven temperature was 250°.

Quantitative studies

A 1-μl portion of the HFB derivative solution was injected into the repor ing

GLC–ECD equipped with an automatic sampler. Quantitative estimation of DE-MCP in the urine samples was accomplished by plotting the area ratios (derivative to internal standard) against the concentration of DE-MCP.

Animal studies

Doses equivalent to 15 and 35 mg/kg of MCP were administered in 0.9% NaCl solution to individual male Wistar rats either intraperitoneally or intravenously through the tail vein. The rats were deprived of food for 12 h before drug administration, but water was allowed *ad libitum*. Cumulative 24-h urine samples were collected, diluted and analysed as described above.

RESULTS AND DISCUSSION

GLC

A representative chromatogram obtained from the extract of rat urine is shown in Fig. 1; no endogenous interference was observed in chromatograms from blank urine samples. Peaks with retention times at 1.98 and 2.35 min were the HFB derivatives of DE-MCP and MCP, respectively. Although base-line resolution between the two peaks was not attained, when a 3% SP 2250 DB column was used, the electronic-integration method provided consistent results, which permitted simultaneous quantitation of both the MCP and DE-MCP peaks.

Linearity was observed in the range studied (0.40–1.85 μg/ml of urine). Quantitation of DE-MCP was accomplished by analysing a serial dilution of known con-

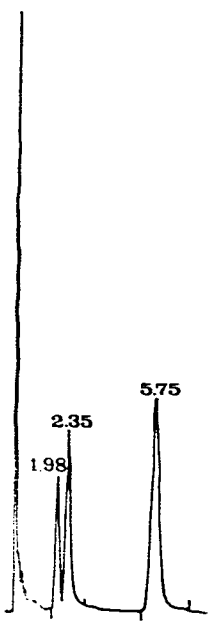


Fig. 1. Representative chromatogram obtained from urine extract. The peaks at 1.98, 2.35 and 5.75 min are the derivatives of DE-MCP, MCP and diazepam, respectively.

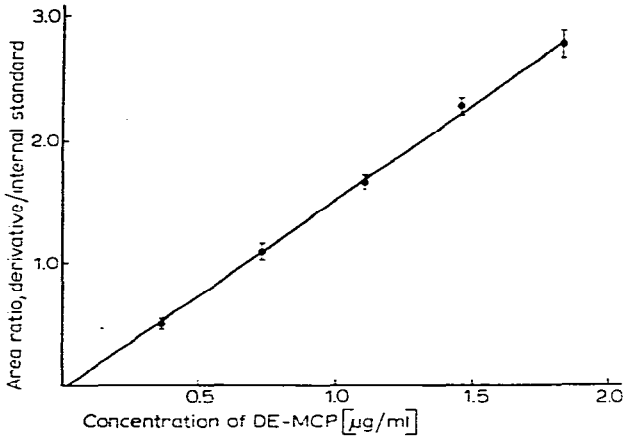


Fig. 2. Standard curve prepared by plotting the area ratios of the HFB derivatives of DE-MCP and diazepam against the concentration of DE-MCP ($r^2 = 0.999$; $n = 5$).

centrations of DE-MCP in the urine extract (see Fig. 2). Regression analysis showed that the best-fit line through the data points was described by:

$$y = 1.535x + 0.039$$

with $r^2 = 0.999$. The HFB derivative was shown to be stable for at least 24 h at ambient temperature.

Removal of excess of derivatizing agent

The presence of trace quantities of HFBA in the derivative solution has been reported to cause spurious peaks and broad solvent fronts[4]. Therefore, development of a method which would remove the excess reagent without diminishing the response of the derivative was necessary. Two methods were suggested by Walle and Ehrsson[4]: one involved drying of the reaction mixture by a gentle stream of nitrogen after incubation, and the other involved hydrolysis of excess of HFBA with water and neutralization with aqueous ammonia. Tam and Axelson[1] have recently reported that the former method decreased the response of the derivative of MCP by at least 67%. Similar results were observed for the derivative of DE-MCP; this was probably due to the volatility of the derivative[1] when the former method was employed.

TABLE I

RECOVERY OF DE-MCP AFTER EXTRACTION FROM RAT URINE

DE-MCP added (ng)	*No. of determinations*	*Recovery after extraction (%)*	*Deviation (%)*
366.8	5	81.43	±6.7
733.6	5	85.25	±5.8
1100	4	86.37	±3.8
1467	5	89.34	±3.7
1834	4	86.49	±4.8
Average		85.78	±4.8

* Each number is the average of a triplicate sample injection into the GLC system.

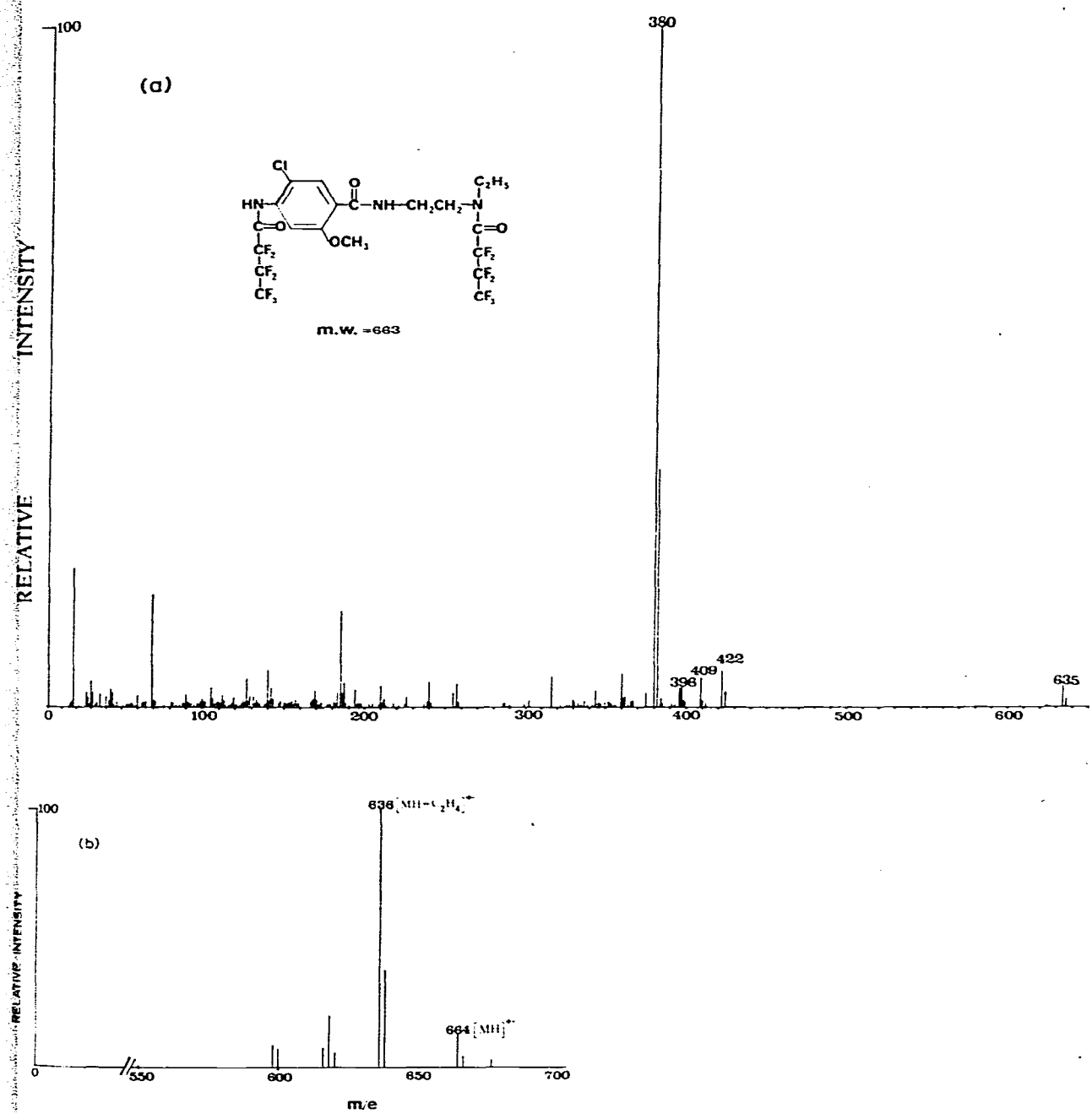


Fig. 3. Mass spectra of the derivative of DE-MCP: (a) EI; (b) CI.

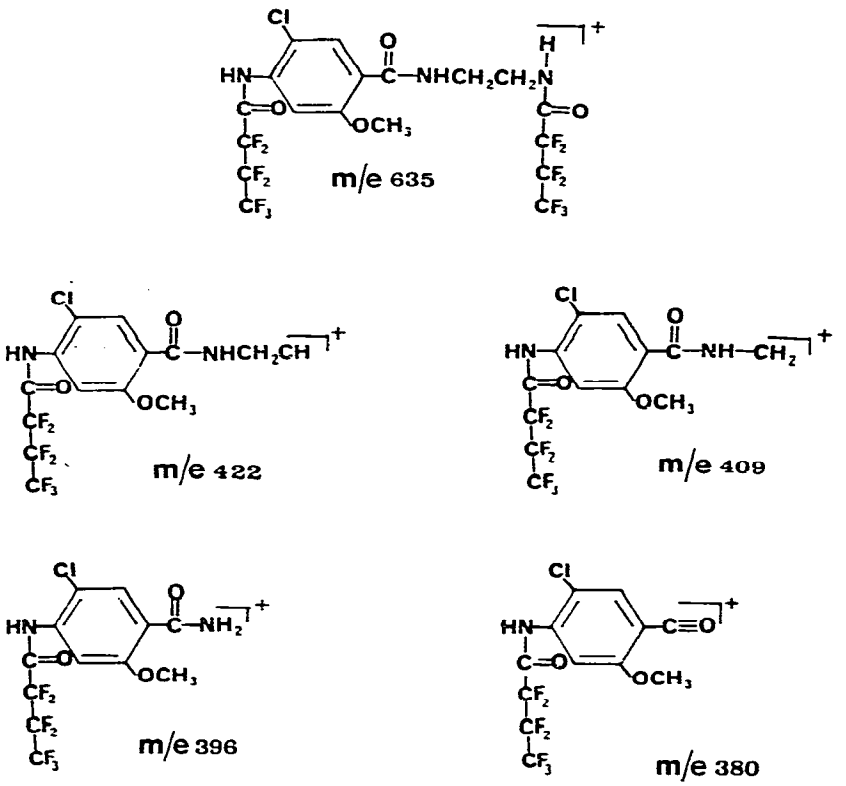
Recovery

The extraction efficiency was evaluated by using a standard curve prepared after dissolving authentic DE-MCP directly in chloroform. Table I shows that the average recovery from urine extracts was 85.78 ± 4.8%.

MS

EI. The fragmentation pattern of the HFB derivative of DE-MCP is similar to that of the HFB derivative of MCP[1]. The base peak is at *m/e* 380 (Fig. 3a); this corresponds to the fragment that cleaved at the carbonyl amide bond (scheme I). The other most intense peaks are at *m/e* 396, 409 and 422. The postulated fragments were described previously[1] and are shown in scheme I. The molecular ion was not detectable, CI-MS being required to study this ion.

CI. As shown in Fig. 3b, the base peak is at *m/e* 636, which corresponds to the removal of the ethylene group at the terminal amine nitrogen. The $(MH)^+$ peak was observed to be at *m/e* 664; this indicates that a di-substituted HFB derivative is formed.



Scheme I. Postulated fragments of derivative of DE-MCP after EI-MS.

Animal data

The applicability of this method was shown by analysing cumulative urine samples collected during the 24 h after drug administration. At the two dose levels studied (15 and 35 mg/kg), the preliminary results (Table II) indicated that different routes of administration of MCP, *viz.*, intravenous and intraperitoneal, have no significant effect on the amounts of MCP and DE-MCP recovered in urine. This implies that first-pass metabolism may not play a major role in reducing the availability of

TABLE II
AMOUNTS OF MCP AND DE-MCP RECOVERED IN URINE AFTER 24 h

Rat	*Weight of rat (g)*	*Dose (mg/kg)*	*Route*	*DE-MCP equivalent to MCP (%)*	*Recovery of MCP (%)*	*Total dose recovered (%)*
A	230	15	Intravenous	12.98	24.81	37.79
B	230	15	Intraperitoneal	16.21	23.72	39.93
C	235	35	Intravenous	9.82	25.03	34.85
D	237	35	Intraperitoneal	12.42	27.39	39.81

MCP as reported earlier[5]. Further studies with more animals to which various dose levels have been administered are required to confirm these results; such studies are currently being carried out.

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