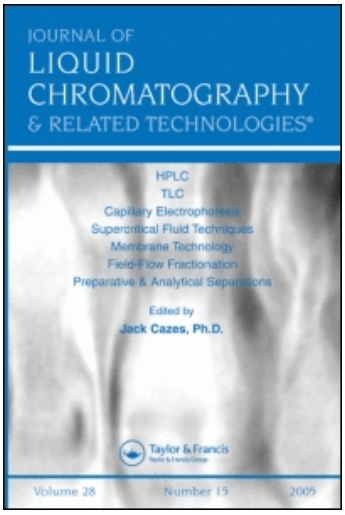

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Simple Isocratic HPLC Method for the Determination of Metoclopramide in Plasma and Urine

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To cite this Article Nygard, Gloria , Lovett, Leslie J. and Khalil, S. K. Wahba(1986) 'A Simple Isocratic HPLC Method for the Determination of Metoclopramide in Plasma and Urine', Journal of Liquid Chromatography & Related Technologies, 9: 1, 157 — 176
To link to this Article: DOI: 10.1080/01483918608076629
URL: http://dx.doi.org/10.1080/01483918608076629

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(1), 157-176 (1986)

A SIMPLE ISOCRATIC HPLC METHOD FOR THE DETERMINATION OF METOCLOPRAMIDE IN PLASMA AND URINE

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ABSTRACT

Metoclopramide concentrations in plasma and urine were determined by high performance liquid chromatography using a cyanopropylsilane column and UV detection. The mobile phase consisted of 0.03M sodium acetate (pH 7.4) and acetonitrile. The plasma samples were extracted with dichloromethane after pH adjustment. Urine proteins were precipitated with acetonitrile. The reproducibility and precision of the methods were demonstrated by the analysis of samples containing 5 - 200 ng/ml plasma and 0.25 - 200 ug/ml urine.

The glucuronide and sulfate conjugates of metoclopramide were also quantitated after differential acid hydrolysis of urine samples. The conditions for acid hydrolysis were studied. The methods have been applied to the analysis of plasma and urine samples obtained from human volunteers.

 0148-3919/86/0901-0157$3.50/0

INTRODUCTION

Metoclopramide (4-amino-5-chloro-2-methoxy-N-[2-diethyl-aminoethyl] benzamide) is an antiemetic drug approved for the control of nausea and vomiting associated with cancer chemotherapeutic drug administration as well as for disorders of gastrointestinal motility. The later action appears to be due to antagonism of dopaminergic mechanisms (1). The drug is rapidly absorbed from the gastro-intestinal tract and excreted in the urine as free and conjugated metoclopramide (2).

Chromatographic methods to determine metoclopramide include thin layer chromatography (3,4), gas chromatography with electron capture (5,6) and mass spectrometric detection (7,8), normal phase HPLC (9-11), and reverse phase HPLC (12,13). These HPLC methods suffer certain limitations including 1) the use of a lengthy extraction procedure, 2) the lack of an internal standard, 3) the use of toxic extraction solvents, 4) the application of the method to plasma or urine only, not both, and 5) limited sensitivity.

The present paper describes metoclopramide analyses in plasma and urine using a high efficiency reverse phase cyanopropylsilane HPLC column. Sample preparation has been minimized by use of a single step extraction for plasma and acetonitrile precipitation of proteins for urine.

Metoclopramide and its glucuronide and sulfate conjugates have each been quantitated in urine samples using the

differential acid hydrolysis procedure of Arita, et al.(14). This method has the disadvantage of requiring a lengthy hydrolysis at 0°C to cleave the glucuronide. The present paper reports a modification of this procedure which shortened the hydrolysis time to seven minutes and therefore was readily applicable to the processing of large numbers of patient samples.

MATERIALS AND METHODS

Instrumentation

A Hewlett Packard Model 1081B liquid chromatograph equipped with an autosampler and a Techsphere-Ultra CN (Phenomenex HPLC Technology, Palos Verdes Estates, CA) column, 150 mm long and 5 mm i.d. A Schoeffel Model GM770 variable wavelength detector was used to monitor the effluent at 280 nm. The signal was recorded on a Hewlett Packard Model 3390A integrator. The degassed mobile phase was pumped through the column at 3 ml/min and the column compartment was maintained at 45°C.

Chemicals and Reagents

Reagent grade dibasic sodium phosphate, hydrochloric acid and sodium hydroxide were used. Methanol, acetonitrile, sodium acetate and dichloromethane were HPLC grade. Metoclopramide hydrochloride was USP grade. Metoclopramide-N-glucuronide and metoclopramide-N-sulfate were synthesized at Colorado State University, Department of Chemistry, Fort Collins, CO. Disopyramide phosphate (Norpace^{R}) was obtained courtesy of G.D. Searle and Co.

Drug Solutions

Plasma assay -- A stock solution of metoclopramide was prepared at 10 mg/10 ml of water (11.8 mg metoclopramide HCl/ 10 ml). Working dilutions of 0.2 and 2.0 ug/ml water were prepared from the stock solution.

Urine assay -- A stock solution of metoclopramide was prepared at 20 mg/10 ml water (23.6 mg metoclopramide HCl/10 ml). A dilution of 0.1 mg/ml water was prepared from the stock solution.

Internal Standard Solution

Plasma assay -- A stock solution of disopyramide phosphate was prepared at 4 mg/50 ml methanol. The extraction solution consisted of a 4 ml aliquot of the stock solution in 1000 ml dichloromethane.

Urine assay -- The precipitation solution was prepared by dissolving 33 mg disopyramide phosphate in a small volume of water and then diluting to 500 ml with acetonitrile.

Mobile Phases

Sodium acetate solution, 0.03M, was prepared in deionized distilled water, and the pH was adjusted to 7.4. The mobile phase for plasma consisted of 35% buffer and 65% acetonitrile. For urine 40% buffer and 60% acetonitrile were used.

Preparation of Plasma Standards

To 2.0 ml of plasma in a 15 ml screw-capped centrifuge tube was added an aliquot (10-200 ul) of drug solution containing

10-400 ng metoclopramide, 0.5 ml Na_2HPO_4 buffer (0.1M, pH 9.5) and 9 ml of extraction solution containing 2.88 ug of the internal standard. The tubes were vortexed for 20 seconds and centrifuged for 10 minutes at 1100xg. The organic layer was then transferred to an evaporating tube (Concentratube^R, Laboratory Research Co., Los Angeles, CA). The sample was evaporated to dryness under a gentle stream of nitrogen at 30°C, reconstituted in 100 ul of methanol and transferred to a polypropylene microvial (P. Weidman & Co., Romanshorn, Switzerland). A 40 ul aliquot was injected onto the column.

Preparation of Urine Standards

To a known volume of urine, an aliquot of metoclopramide solution was added to prepare standards containing 0.25-200 ug/ml urine. A 200 ul aliquot of the spiked urine was then placed in a 10x75 mm pyrex tube, and the proteins were precipitated with 350 ul acetonitrile containing 23.1 ug of the internal standard. The tubes were vortexed for 10 seconds and centrifuged for 10 minutes at 1100xg. An aliquot of the supernatant was transferred to a polypropylene microvial before injection of 30 ul into the chromatograph.

Quantitation

Standard curves for plasma and urine were constructed utilizing three replicates at each concentration. The peak heights were measured and the ratios of drug to internal standard were plotted against concentration (ng or ug metoclopramide/ml plasma or urine).

Recovery

Plasma assay -- Spiked samples containing known concentrations of metoclopramide were carried through the analysis and the peak heights of drug and internal standard were measured. An equivalent amount of metoclopramide was added to 9 ml of extraction solution, vortexed, evaporated to dryness and reconstituted in methanol for injection into the HPLC. The peak heights of the drug and internal standard were measured for comparison with the extracted samples to estimate percent recovery from extraction.

Urine assay -- Spiked samples containing known concentrations of metoclopramide in urine or water were carried through the analysis. The peak height ratios were compared to estimate percent recovery after protein precipitation.

Analysis of Patient Plasma Samples

Plasma samples from patients receiving metoclopramide were analyzed using the procedure described above for plasma standards. The amount of drug in patient samples was calculated by comparison to a standard curve prepared daily.

Analysis of Patient Urine Samples

A differential hydrolysis procedure was used to quantitate metoclopramide and its glucuronide and sulfate conjugates. Each sample was processed three different ways:

Treatment A -- To quantitate free metoclopramide, a 200 ul aliquot of the urine sample was combined with 350 ul

acetonitrile containing internal standard. The sample was vortexed, centrifuged and the supernatant injected into the HPLC.

Treatment B -- To quantitate metoclopramide glucuronide, a 200 ul aliquot of urine was incubated for seven minutes with 100 ul 1M HCl at room temperature. The hydrolysis reaction was stopped by addition of 50 ul 2M NaOH and 350 ul acetonitrile containing the internal standard. The samples were vortexed, centrifuged and chromatographed. The increase in metoclopramide from treatment A to B represented the concentration of hydrolyzed metoclopramide glucuronide.

Treatment C -- To quantitate metoclopramide sulfate, a 200 ul aliquot of urine was incubated for five minutes with 100 ul 1M HCl at 100°C in an aluminum heating block. The samples were then treated with 50 ul 2M NaOH and 350 ul precipitation solution and processed as above. The increase in metoclopramide concentration from treatment B to C is due to the hydrolyzed sulfate conjugate.

RESULTS AND DISCUSSION

The chromatographic conditions were chosen to give good separation of metoclopramide and the internal standard from endogenous peaks in plasma and urine as well as from the metabolites in the urine. The use of a high efficiency 5 micron particle size cyanopropylsilane column in a reversed phase mode with a mobile phase of acetonitrile and sodium

acetate (0.03M, pH7.4) produced excellent separation of all components. The urine samples were eluted with a slightly lower percentage of acetonitrile to allow baseline separation of the drug from the solvent front constituents of urine. The pure metoclopramide glucuronide and sulfate metabolites eluted earlier than the parent drug. A late eluting peak endogenous to urine had a retention time more than twice that of the internal standard, but with the judicious setting of "stop" time and use of an autosampler successive injections could be made without interference. Typical chromatograms of plasma and urine samples are found in Figures 1 and 2, respectively.

The plasma assay method described here has a lower limit of sensitivity (1 ng/ml plasma) than those reported in the literature (2.5 ng/ml or higher) (3-13). Previous methods used either normal phase silica columns or reverse phase C8 or C18 columns. Cyano columns are well suited to the analysis of basic compounds and are commonly used for basic drugs such as the tricyclic antidepressants (15) and antiarrhythmics (16).

Metoclopramide is readily extracted from plasma in a single step using dichloromethane at alkaline pH. The percent recovery for the method described here was found to be 87%. It is not necessary to extract urine samples since they contain a much higher concentration of drug. Protein precipitation of urine with acetonitrile containing the internal standard was

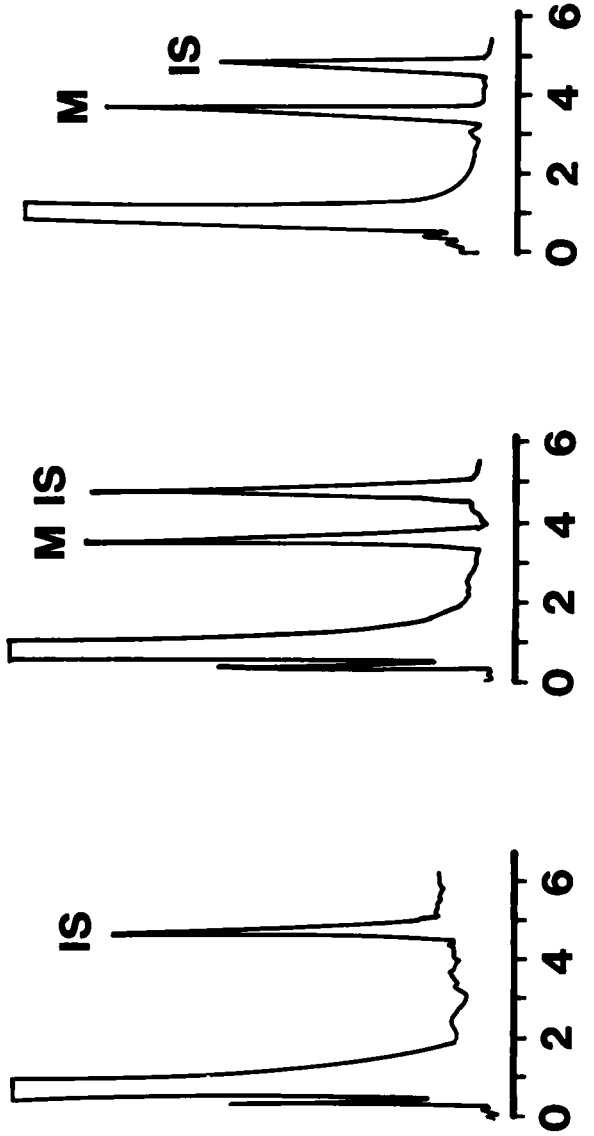

Figure 1. Typical Chromatograms of Plasma Extracts

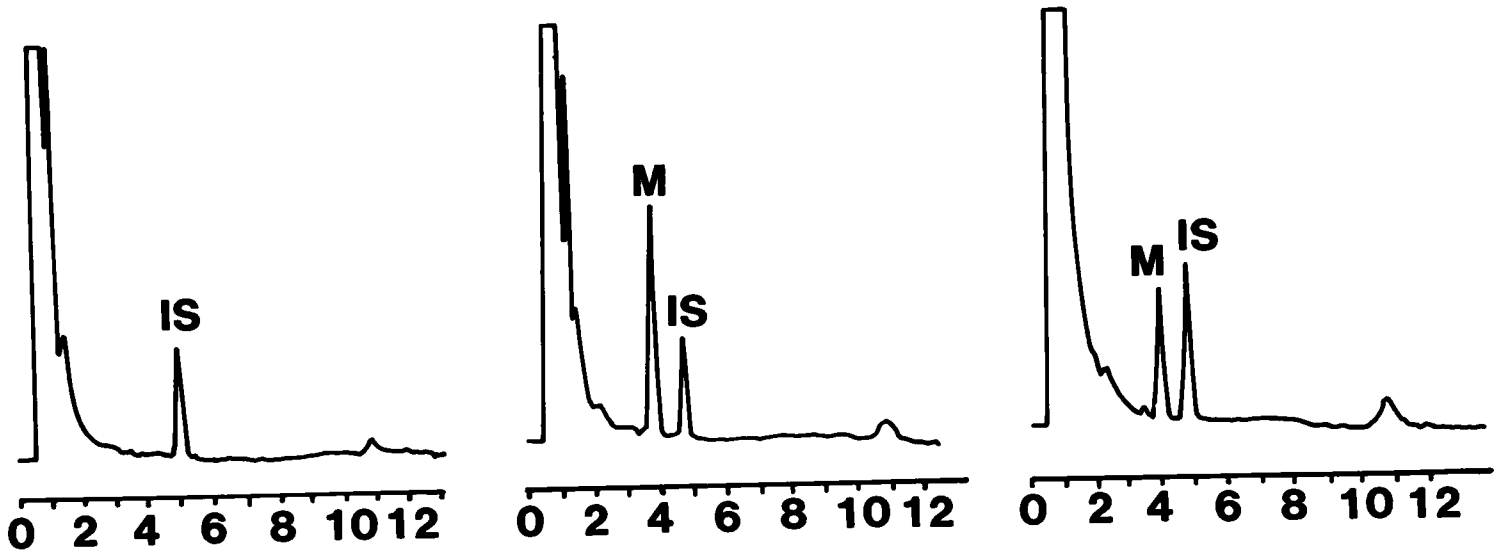

Figure 2. Typical Chromatograms of Urine Samples

chosen for the current method. This simple sample processing lends itself well to the handling of large numbers of urine samples including those from differential acid hydrolysis. The use of acetonitrile for precipitation of the urine proteins has the added advantage of being the organic modifier in the mobile phase and results in high recovery of metoclopramide. The recovery of metoclopramide from urine was quantitative at all the concentrations of the standard curve. The lower limit of sensitivity for the urine assay was 0.1 ug/ml urine.

The ratio of the peak height of the drug to the peak height of the internal standard was calculated at each concentration for both plasma and urine. Statistical analysis of the data by linear regression indicated linearity and reproducibility in the range of 5-200 ng/ml plasma and 0.25-200 ug/ml urine (Tables 1 and 2).

The differential urine hydrolysis method used here was modified from that of Arita, et al. (14). That procedure specified incubation of urine samples for up to 90 minutes at 0°C in order to cleave metoclopramide glucuronide. This lengthy step was cumbersome for analysis of large numbers of urine samples, so the method was modified to use room temperature incubation. Urine samples containing a known concentration of the glucuronide were mixed with acid and allowed to incubate for various times. The hydrolysis reactions were stopped by the addition of base and analyzed for

TABLE 1. LINEARITY AND PRECISION OF METOCLOPRAMIDE PLASMA ASSAY METHOD

x = Concentration of Metoclopramide Added (ng/ml plasma)	y = Concentration of Metoclopramide Found (ng/ml plasma)*	CV (%)
5	6.88 ± 0.78	11.3
10	11.35 ± 0.87	7.7
20	20.80 ± 1.02	4.9
30	29.94 ± 1.46	4.9
50	49.95 ± 1.24	2.5
100	95.04 ± 2.88	3.0
200	202.30 ± 7.88	3.9

N = 3

R = 0.9985

Regression equation: $y = m x + b$

$y = 0.0236 x = 0.0079$

*mean ± standard deviation

TABLE 2. LINEARITY AND PRECISION OF METOCLOPRAMIDE URINE ASSAY METHOD

x = Concentration of Metoclopramide Added (ug/ml urine)	y = Concentration of Metoclopramide Found (ug/ml urine)*	CV (%)
0.25	0.309 ± 0.021	6.80
0.5	0.476 ± 0.046	9.66
1	0.952 ± 0.052	5.46
2.5	2.524 ± 0.006	0.24
5	4.858 ± 0.236	4.86
10	9.483 ± 0.168	1.77
25	26.204 ± 0.677	2.58
50	51.276 ± 1.836	3.58
100	103.026 ± 2.979	2.89
200	198.051 ± 13.879	7.01

N = 3

R = 0.9980

Regression equation: $y = m x + b$

$y = 0.2688 x + 0.0034$

*mean ± standard deviation

TABLE 3. HYDROLYSIS OF METOCLOPRAMIDE GLUCURONIDE AND SULFATE CONJUGATES IN URINE QUALITY CONTROL SAMPLES AT AMBIENT TMPERATURE

Hydrolysis Time (min)	Concentration Metoclopramide After Hydrolysis of Glucuronide Conjugate (ug/ml urine)	Concentration Metoclopramide After Hydrolysis of Sulfate Conjugate (ug/ml urine)
0	0	0.30 *
0.5	0.21	0.28
1	0.64	0.25
1.5	0.91	0.29
2	1.04	0.25
3	1.28	0.26
5	1.47	0.29
7	1.61	0.34
9	1.59	0.36
11	1.57	0.35
15	1.54	0.37

N = 2

* Metoclopramide contaminant in sulfate conjugate from synthesis

metoclopramide. The results show the glucuronide hydrolysis to be complete within seven minutes (Table 3) at ambient temperature. Urine samples containing metoclopramide sulfate were tested in the same way to determine after what time a detectable decomposition of that conjugate might occur under room temperature hydrolysis conditions. That experiment showed the sulfate conjugate to be stable for at least 15 minutes under these conditions (Table 3). The room temperature hydrolysis time specified is therefore sufficient for complete hydrolysis of metoclopramide glucuronide without

decomposition of the sulfate conjugate. Arita, et al. obtained complete hydrolysis of metoclopramide sulfate at 100°C between two and six minutes, and the present study confirmed this.

The plasma and urine assay methods have been applied to the analysis of samples obtained from volunteers taking metoclopramide. A typical plasma kinetic profile of metoclopramide in a volunteer receiving a 20 mg oral dose is shown in Figure 3. The corresponding urine excretion data for the drug and its conjugates is plotted in Figure 4. Quality control samples were analyzed each day and the methods showed good within-run and day-to-day reproducibility. The plasma controls were spiked with two concentrations of metoclopramide (Table 4). The urine controls were of four types -- spiked with the drug and its conjugates separately and spiked with a mixture of all three (Tables 5 A,B,C). The

TABLE 4. REPRODUCIBILITY OF THE METOCLOPRAMIDE PLASMA ASSAY

	Sample	Average Concentration of Metoclopramide (ng/ml plasma)	S.D.	CV(%)	N
Within-Run	QC low	18.47	0.47	2.56	3
	QC high	46.37	0.21	0.45	3
Day-to-Day	QC low	18.72	0.16	0.88	5
	QC high	46.70	0.54	1.15	5

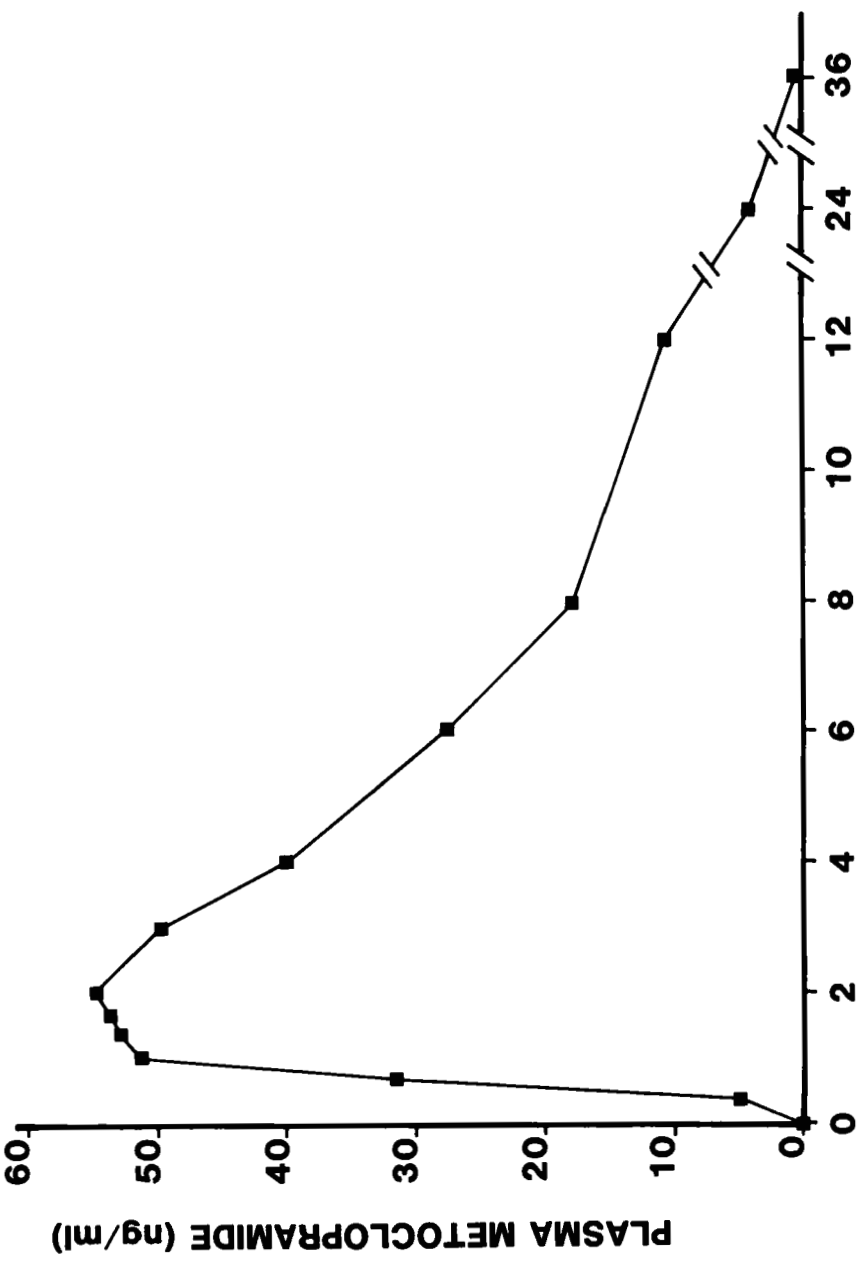

Figure 3. Plasma Concentration of Metoclopramide After a 20 mg Oral Dose

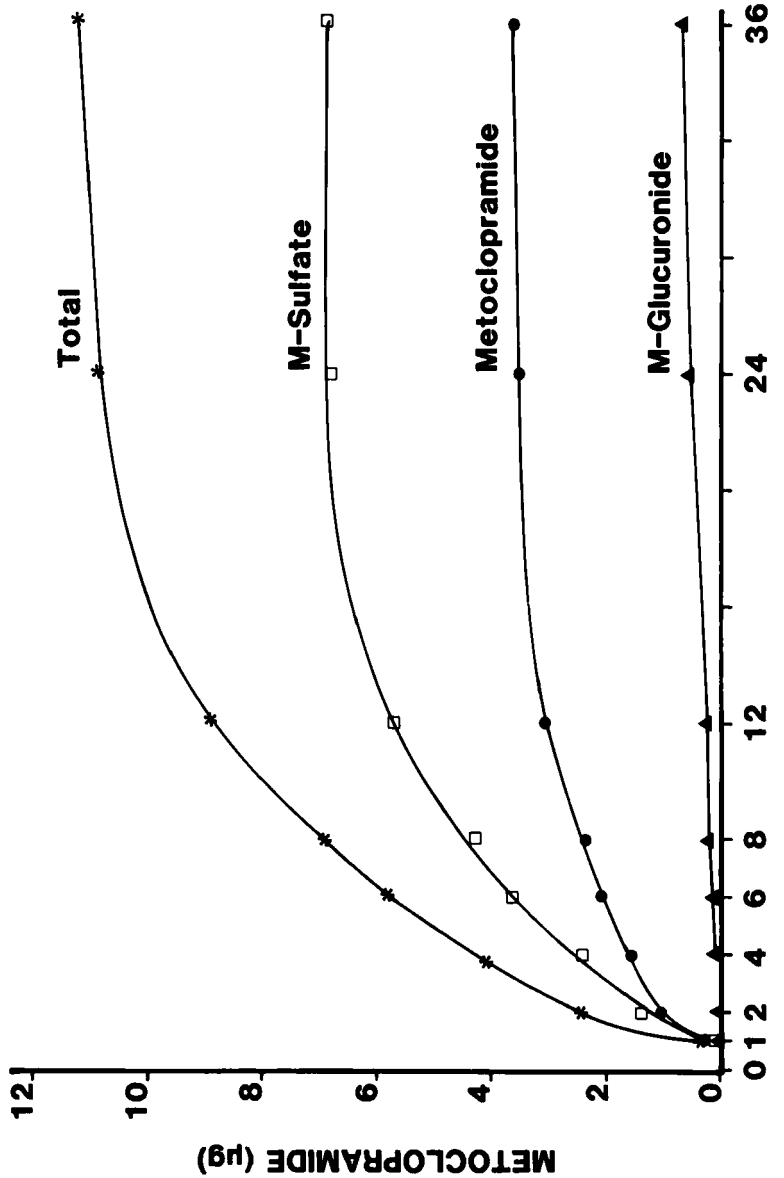

Figure 4. Cumulative Urine Excretion of Metoclopramide and the Glucuronide and Sulfate Conjugates After a 20 mg Oral Dose

TABLE 5A. REPRODUCIBILITY OF THE METOCLOPRAMIDE URINE ASSAY WITHOUT ACID HYDROLYSIS (TREATMENT A)

	Drug(s) Added	Average Concentration of Metoclopramide (ug/ml urine)	S.D.	CV(%)	N
Within-Run	M	4.1	0.2	4.9	4
	MG	--	--	--	--
	MS	0.35*	0.06	17.1	4
	M+MG+MS	4.5	0.3	6.7	4
Day-to-Day	M	4.1	0.1	2.4	5
	MG	--	--	--	--
	MS	0.34*	0.05	14.7	5
	M+MG+MS	4.3	0.3	7.0	5

* Metoclopramide contaminant in sulfate conjugate from synthesis

TABLE 5B. REPRODUCIBILITY OF THE METOCLOPRAMIDE URINE ASSAY WITH HYDROLYSIS AT AMBIENT TEMPERATURE (TREATMENT B)

	Drug(s) Added	Average Concentration of Metoclopramide (ug/ml urine)	S.D.	CV(%)	N
Within-Run	M	4.1	0.2	4.9	4
	MG	1.5	0.1	6.7	4
	MS	0.28*	0.09	32.1	4
	M+MG+MS	6.2	0.2	3.2	4
Day-to-Day	M	4.1	0.1	2.4	5
	MG	1.6	0.1	6.3	5
	MS	0.34*	0.05	14.7	5
	M+MG+MS	6.1	0.2	3.3	5

* Metoclopramide contaminant in sulfate conjugate from synthesis

TABLE 5C. REPRODUCIBILITY OF THE METOCLOPRAMIDE URINE ASSAY WITH HYDROLYSIS AT 100°C (TREATMENT C)

	Drug(s) Added	Average Concentration of Metoclopramide (ug/ml urine)	S.D.	CV(%)	N
Within-Run	M	4.1	0.1	2.4	4
	MG	1.6	0.1	6.3	4
	MS	3.9	0.1	2.6	4
	M+MG+MS	9.6	0.4	4.2	4
Day-to-Day	M	4.0	0.2	5.0	5
	MG	1.5	0.1	6.7	5
	MS	3.8	0.1	2.6	5
	M+MG+MS	9.6	0.1	1.0	5

sulfate conjugate contained a low but detectable quantity of metoclopramide, apparently a contaminant from its synthesis.

CONCLUSIONS

The methods for plasma and urine metoclopramide analysis presented in this paper have several advantages over the procedures described in the literature. The plasma assay provides greater sensitivity than methods now available and has good linearity and reproducibility. The assay for urine metoclopramide uses a simple acetonitrile protein precipitation step instead of extraction, and yet has equivalent sensitivity to other procedures. The method is linear and reproducible.

The present paper presents a modified differential acid hydrolysis procedure for quantitation of the glucuronide and sulfate conjugates of metoclopramide. The method shortens

the incubation time several fold and uses both ambient and elevated temperatures. The sensitivity, simplicity, reproducibility and linearity of both the plasma and urine assays make them applicable for monitoring therapeutic metoclopramide levels in patients or for pharmacokinetic studies.

ACKNOWLEDGEMENTS

The authors wish to thank G.D. Searle and Co. for providing a free authentic sample of Norpace^{R}.

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