Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, Nos 10–12, pp. 935–940, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00
Pergamon Press plc

Column-switching liquid chromatographic determination of ML-1035 sulphoxide and its sulphone and sulphide metabolites in rat urine*

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Abstract: A fully automated column-switching LC assay has been developed for the simultaneous determination of a gastroprokinetic agent, ML-1035 sulphoxide, and its sulphone and sulphide metabolites in rat urine. ML-1035 Sulphoxide is a metoclopramide analogue. The method involved direct injection of a diluted urine sample into a CN extraction column for sample clean-up. Polar urine components, including proteins, were flushed to waste. The retained compounds were then eluted onto a C_8 analytical column for further separation and analysis by fluorescence detection. After the subsequent washing and re-equilibration with a sequence of three solvent mixtures, the extraction column was ready for the next injection. The recovery of the compounds from the extraction column was 85–90%. The limit of quantitation for all compounds of interest was 25 ng ml^{-1} or lower, using a 100 μl specimen of urine. Good inter-day precision (2.1–10.0%), accuracy (0.3–18.0%), and linearity were obtained for all compounds over a range of 25–1000 ng ml^{-1}. The applicability of the LC method was validated with urine samples from rats that had received ML-1035 sulphoxide.

Keywords: *Column-switching; HPLC; metoclopramide analogues (ML-1035); gastroprokinetic agents; antiemetic agents.*

Introduction

ML-1035, 4-amino-5-chloro-2-[2-(methylsulphinyl)ethoxy]-*N*-[2-(diethylamino)ethyl] benzamide, was synthesized as a structural modification of metoclopramide and has been identified as a new gastroprokinetic agent. Metoclopramide has been in wide clinical use as an anti-emetic and as a stimulant of upper gut motility for more than a decade [1–3]. ML-1035 is a sulphoxide compound (Fig. 1) that has been shown in an urinary excretion study in rats to undergo reduction and oxidation to form the sulphide and sulphone metabolites, respectively.

Column-switching LC with direct injection of biological fluids onto a short extraction column has gained much attention since the technique was reported a decade ago by Roth *et al.* [4]. For the analysis of ML-1035 sulphoxide and its sulphone and sulphide metabolites, an automated column-switching LC method has been developed and validated. This method utilized direct injection of diluted rat urine onto a short extraction column for sample clean-up, followed by elution onto an analytical column for further analysis. This automated LC procedure provided a high degree of sensitivity, reproducibility, and rapid sample throughput. The method has been used

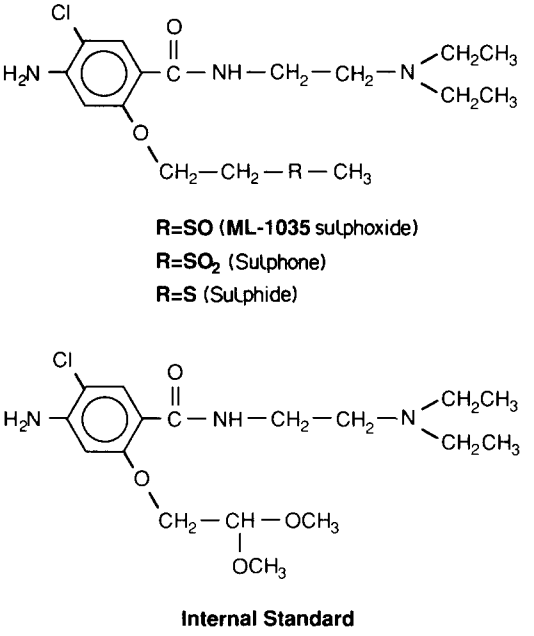

Figure 1
Chemical structures of ML-1035 sulphoxide, sulphone, sulphide and the internal standard used in the LC assay.

* Presented at the "Third International Symposium on Pharmaceutical and Biomedical Analysis", April 1991, Boston, MA, USA.

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to determine the excretion and metabolic profiles of ML-1035 sulphoxide in rat urine.

Experimental

Materials and reagents

HPLC grade acetonitrile and isopropyl alcohol were obtained from Burdick and Jackson (Muskegon, MI, USA). Sodium dodecyl sulphate (SDS) of electrophoresis grade was obtained from Bio-Rad (Richmond, CA, USA). Triethylamine (>99%) was from Aldrich Chemical Co. (Milwaukee, WI, USA). Tetrahydrofuran was from J.T. Baker (Phillipsburg, NJ, USA). Sodium phosphate, monobasic, was from Mallinckrodt Co. (Paris, KY, USA). The internal standard and the ML-1035 sulphoxide, sulphone and sulphide analogues (Fig. 1) were provided by Marion Merrell Dow Inc. (Kansas City, MO, USA). The acrodisc filters (0.2 μm) were purchased from Gelman Sciences (Ann Arbor, MI, USA).

Solutions and sample preparation

A 1.0 mg ml^{-1} stock mixture of ML-1035 sulphoxide, sulphone and sulphide was made in methanol. The stock solution was diluted serially with 0.5 M phosphate buffer (pH 4.3) and used to prepare rat urine standards with concentrations of 25, 50, 75, 100, 200, 400, 600, 800, and 1000 ng ml^{-1}. Urine samples were first centrifuged at 1500**g** for 15 min to obtain particulate-free urine. Urine standards or unknown urine samples (1 ml) from the *in vivo* study were mixed with 25 μl of 10 μg ml^{-1} internal standard. Each of these samples was then diluted to 2 ml with 0.5 M phosphate (monobasic) at pH 4.3, vortexed briefly, and passed through a 0.2 μm acrodisc filter. A 200 μl injection of the resulting mixture was made into the LC system.

Columns and mobile phases

The LC column system consisted of an extraction column and an analytical column. The extraction column was a Resolve CN precolumn (4 mm × 4.6 mm, 5 μm) from Waters (Milford, MA, USA) that was used to retain the test compounds. A Spherisorb C_8 column (150 mm × 4.6 mm, 5 μm) (Alltech Associates, Deerfield, IL, USA) was used to analyse the test compounds after elution from the extraction column. The guard column was a Hypersil MOS column (10 mm × 4.6 mm, 5 μm) from Alltech Associates. Four different mobile phases were used: M1, 32.5% acetonitrile in 50 mM NaH_2PO_4 containing 0.25% SDS and 0.2% triethylamine; M2, 0.5% SDS in 50 mM NaH_2PO_4; M3, 0.25% SDS and 2% acetonitrile in 50 mM NaH_2PO_4; and M4, acetonitrile–isopropanol–tetrahydrofuran (69.9:30:0.1, v/v/v). All mobile phases were degassed with helium prior to use. The extraction column was replaced every 100–150 injections. The guard column was changed after 200–300 injections. The analytical column had a lifetime of more than 2000 injections.

Instrumentation

Figure 2 is a schematic representation of the column-switching LC. Pump A (Waters Model 600 Multisolvent Delivery System; Waters, Milford, MA, USA) delivered the three purging solvent mixtures, M2, M3, and M4. Pump B (Waters Model 510) delivered the elution buffer M1. The samples were injected with the Waters autosampler (Model 700 Satellite WISP). An electrically driven six-port switching valve (Autochrom, Ann Arbor, MI, USA) was used to route the extracted compounds onto the analytical column. The autosampler, the switching valve, and the purging events were controlled by the Waters 600E System Controller and the NEC Power Mate-2 Computer (Waters, Milford, MA, USA). Detection was by means of an LS-40 fluorescence detector (Perkin–Elmer, Norwalk, CT, USA) at excitation 308 nm and emission 350 nm. The Beckman Peak Pro System was used to collect,

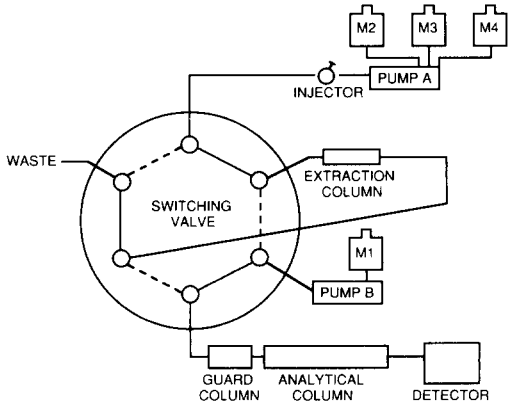

Figure 2
Flow scheme for a column-switching LC system. M1 to M4 are four different mobile phases. The time events associated with the valve switching and solvent delivery are detailed in the Experimental section. Injection mode: —; elution mode: – – –.

integrate, and analyse the chromatographic data.

Automated analytical procedure

The LC analysis was conducted at room temperature. One run of the automated urine analysis required 30 min and included the following steps.

Step A (0–2.5 min, injection mode). A 200 µl aliquot of the diluted (1:1) urine sample was injected into the extraction column for sample clean-up. Proteins and other polar components were flushed to waste with M2 at 2 ml min, while lipophilic compounds, including the compounds of interest, were retained on the extraction column.

Step B (2.5–5.0 min, elution mode). At 2.5 min, the switching valve was automatically rotated to the elution mode. The retained drug compounds were then eluted with M1 at 2 ml min^{-1} from the extraction column onto the analytical column for further analysis. Note that this is a forward-flush pattern.

Step C (5.0–9.0 min, injection mode). At 5.0 min, the switching valve was rotated back to the injection mode. The extraction column was then purged with M3 at 2 ml min^{-1}.

Step D (9.0–12.0 min, injection mode). The extraction column was purged with M4 at 2 ml min^{-1} to remove any strongly retained endogenous hydrophobic components.

Step E (12.0–15.0 min, injection mode). The extraction column was flushed with M3 at 1 ml min^{-1}, which served as a buffer between M4 and M2.

Step F (15.0–30.0 min, injection mode). The extraction column was reconditioned with M2 at 1 ml min^{-1}. At the end of Step E, the chromatographic data were processed and the LC system was ready for the next injection. Quantitation of ML-1035 sulphoxide and its metabolites was based on the peak height ratios.

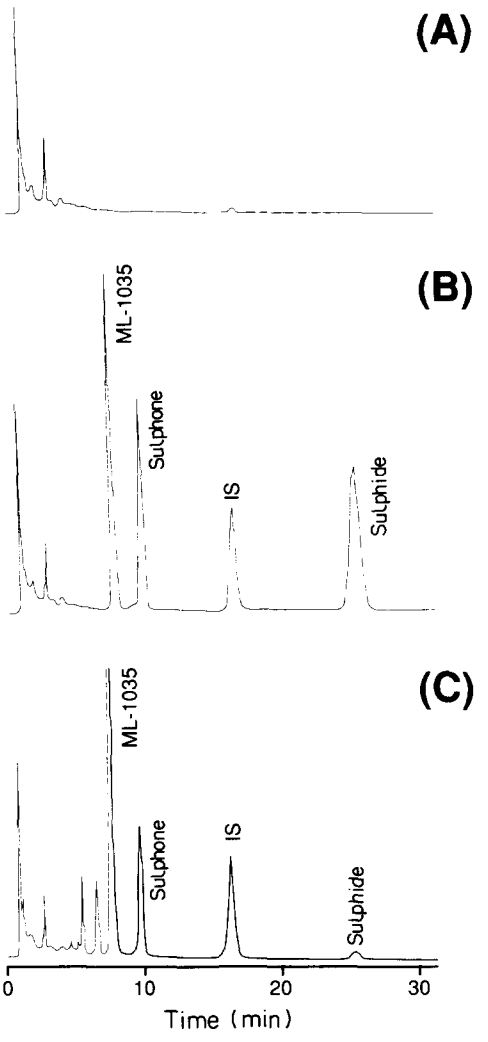

Figure 3
Typical chromatograms: (A) rat urine; (B) rat urine spiked with 600 ng ml^{-1} each of ML-1035 sulphoxide, the sulphone, the sulphide and 250 ng ml^{-1} internal standard (IS); (C) a urine sample collected between 24 and 48 h from a rat that received an intravenous dose of 10 mg kg^{-1} ML-1035 sulphoxide.

Results and Discussion

Specificity and linearity

ML-1035 sulphoxide, sulphone and sulphide, and the internal standard in rat urine were well-separated under the experimental conditions. Panels A and B of Fig. 3 indicate that there were no interfering peaks from the rat urine. Demonstrated in Panel C is a urine sample collected from a rat between 24 and 48 h after an intravenous dose of ML-1035 sulphoxide. In addition to the sulphone and the sulphide metabolites that were formed after dosing, two unknown peaks eluted earlier than ML-1035 sulphoxide. Their identities have yet to be investigated.

The peak height ratios of analyte:IS were linearly related within a concentration range of 25–1000 ng ml^{-1} for ML-1035 sulphoxide and

the two metabolites, with a correlation coefficient greater than 0.999 for the three compounds. Linear equations for the concentration range were: $y = 0.0056x + 0.0072$, $y = 0.0034x - 0.0073$, and $y = 0.0023x - 0.0274$ for ML-1035 sulphoxide, sulphone, and sulphide, respectively. The limit of quantitation for ML-1035 sulphoxide, the sulphone and the sulphide was 25 ng ml^{-1} or lower using a 200 μl injection of diluted rat urine.

Extraction efficiency

The efficiency of the extraction column was evaluated by comparing the peak heights of the tested compounds in rat urine under column-switching conditions to those of the tested compounds in 0.5 M phosphate buffer (pH 4.3) that were directly injected onto the analytical column. As shown in Table 1, the recoveries from the extraction column for ML-1035 sulphoxide, sulphone and sulphide at four different concentrations were estimated to be 88.6, 85.4 and 89.2%, respectively.

Precision and accuracy

The within-run precision and accuracy of the method was examined at three concentrations. These results are shown in Table 2. Over the range 25–1000 ng ml^{-1}, the precision of the assay was excellent with the RSD ranging from 0.6 to 3.1%, 0.8 to 3.3%, and 1.6 to 2.8% for ML-1035 sulphoxide, sulphone and sulphide, respectively. Tested at the same concentrations, the day-to-day precisions were excel-

Table 1
Recovery of ML-1035 sulphoxide, the sulphone and the sulphide in rat urine from the extraction column

Concentration (ng ml^{-1})	ML-1035 sulphoxide (%)	Sulphone (%)	Sulphide (%)
25	86.8	82.9	80.5
100	90.8	85.1	95.1
400	89.3	83.4	91.0
1000	87.5	90.3	90.0
Mean ± SD	88.6 ± 1.8	85.4 ± 3.4	89.2 ± 6.2

The data were obtained by comparing peak heights of each compound generated under column-switching conditions to those heights produced after direct injection of compounds in phosphate buffer onto the analytical column. The recovery at each concentration was the average of four replicate experiments.

Table 2
Within-run precision and accuracy of the assay

Predicted conc. (ng ml^{-1})	Observed mean concentration (RSD) (% error)		
	ML-1035 sulphoxide	Sulphone	Sulphide
25	24.0 (0.8) (3.9)	24.6 (3.3) (1.8)	27.3 (2.8) (9.1)
100	102.7 (1.5) (2.7)	103.5 (2.7) (3.5)	104.0 (2.1) (4.0)
400	397.5 (3.1) (0.6)	395.5 (2.7) (1.1)	390.3 (2.2) (2.4)
1000	1000.7 (0.6) (0.1)	1000.5 (0.8) (0.2)	1003.6 (1.6) (0.4)

Precision and accuracy are expressed as RSD and percentage error, respectively. Each data point was the result of three replicate experiments.

Table 3
Between-run precision and accuracy of the assay

Predicted conc. (ng ml^{-1})	Observed mean concentration (RSD) (% error)		
	ML-1035 sulphoxide	Sulphone	Sulphide
25	24.2 (10) (3.3)	26.3 (6.9) (5.0)	29.6 (3.6) (18)
100	109.1 (4.8) (9.5)	108.8 (2.1) (8.8)	107.5 (2.9) (7.5)
400	395.9 (5.9) (1.0)	391.1 (3.5) (2.2)	379.9 (3.0) (5.0)
1000	1002.8 (3.8) (0.3)	1006.4 (3.0) (0.6)	1013.5 (2.3) (1.4)

Precision and accuracy are expressed as RSD and percentage error, respectively. Each data point was obtained on three different occasions. Duplicate experiments were performed at each occasion.

lent with the RSD ranging from 3.8 to 10%, 2.1 to 6.9%, and 2.3 to 3.6% for ML-1035 sulphoxide, sulphone and sulphide, respectively (Table 3). When compared to the predicted concentrations, the observed concentrations of the tested compounds also reflected that the accuracy of the method was satisfactory.

Stability tests

The stability of the urine samples and the extraction column was examined. The stability of the samples at room temperature was tested by making 55 consecutive injections of the same urine sample over a period of approximately 32 h. As demonstrated in Fig. 4, there were no significant changes in the peak height ratios between the first five and the last five injections. In fact, there was no significant changes in the chromatograms after the consecutive injections (Fig. 5). This indicated that the drug and its metabolites in the buffered urine were stable at room temperature during the test period. In addition, urine samples spiked with the test compounds were stable for more than 3 months at −40°C (data not shown).

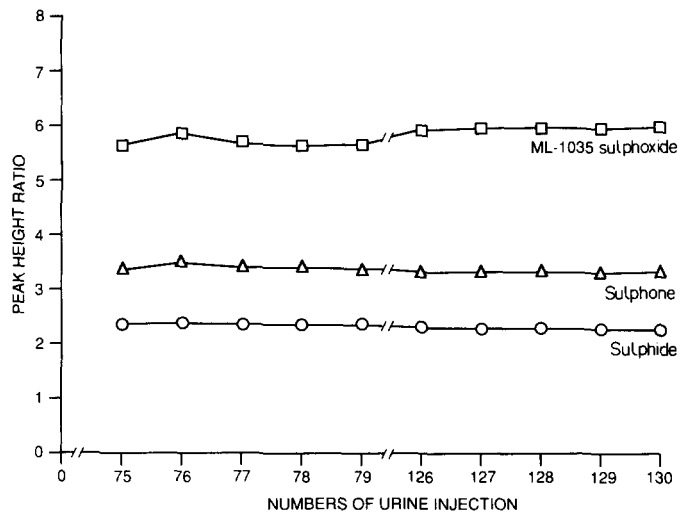

Figure 4
Peak height ratios obtained from the first five and the last five urine samples of 55 consecutive injections over a period of approximately 32 h. Each compound was spiked into rat urine at 1000 ng ml^{-1}.

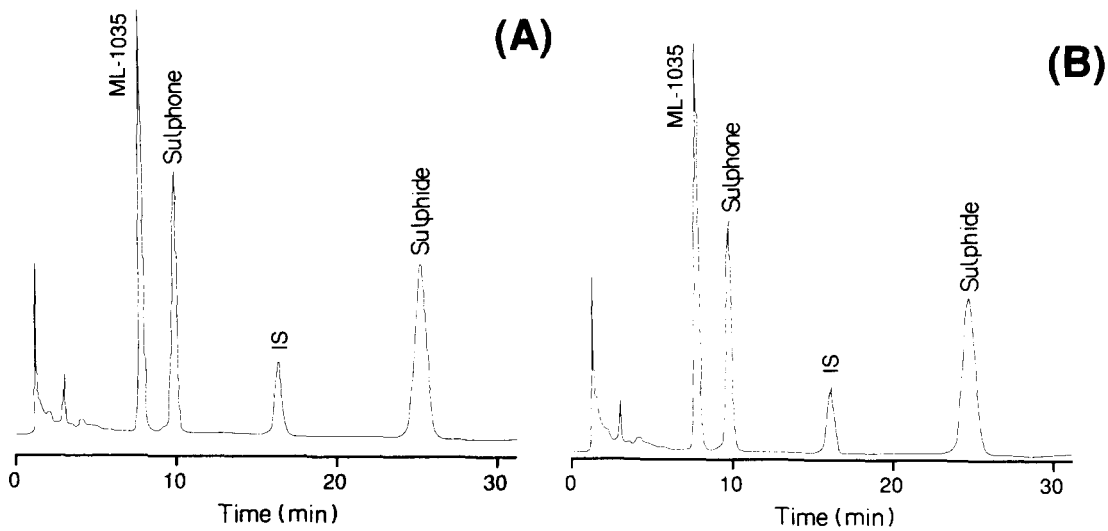

Figure 5
Two representative chromatograms showing the reproducibility of the same urine sample at room temperature after 55 consecutive injections. (A) and (B) are the corresponding chromatograms of the 75th and 130th injection, respectively, as shown in Fig. 4. Urine was spiked with ML-1035 sulphoxide, the sulphone and the sulphide at 1000 ng ml^{-1} each, and with the IS at 250 ng ml^{-1}.

The stability study results (Figs 4 and 5) also indicate the extraction column was performing extremely well even after a large number of injections. In fact, the same extraction column had been injected with 74 different urine samples before the first sample of the stability study was injected. These results suggest that the extraction column could endure more than 130 injections of urine samples before replacement was needed. The Guard-Pak pre-columns used for sample extraction are short and inexpensive, and incorporate a radial distribution device that spreads the injected sample over the frit. This allows the retained compounds to adhere to a narrow band and reduces the likelihood the frits will become clogged after a number of injections of biological fluids [5, 6].

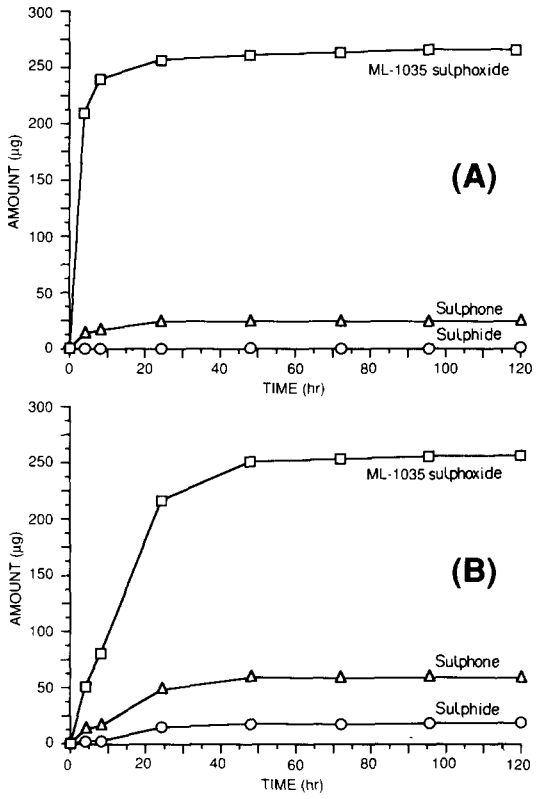

Figure 6
Cumulative urinary excretion profiles of ML-1035 sulphoxide, the sulphone and the sulphide in rats receiving (A) an intravenous dose of 10 mg kg^{-1} and (B) an oral dose of 60 mg kg^{-1}.

Method application

The present LC method has been used to assay rat urine samples obtained from urinary excretion studies. Figure 6 shows the cumulative urinary excretion profile of ML-1035 sulphoxide, sulphone and sulphide in two rats that received (A) an intravenous does of 10 mg kg^{-1} and (B) an oral dose of 60 mg kg^{-1} ML-1035 sulphoxide. These preliminary results suggest that in rats, the sulphone is the major metabolite and that a higher concentration of the metabolites were generated after oral administration. These results also indicate that the present column-switching LC technique is very useful for urinary excretion studies on ML-1035 sulphoxide, and the drug and its sulphone and sulphide metabolites can be quantitated in the urine. The method can also be adapted to analyse ML-1035 sulphoxide and its metabolites in dog and human urine.

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[Received for review 29 April 1991; revised manuscript received 23 May 1991]