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Short Communication

Quantitation of a novel antiemetic (ADR-851) in plasma and urine by reversed-phase high-performance liquid chromatography with fluorescence detection[a]

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ABSTRACT

A sensitive and specific bioanalytical method for quantitation of a novel antiemetic (ADR-851) in plasma and urine has been developed and validated. The drug and internal standard (metoclopramide) are extracted from the plasma matrix by solid-phase extraction on cyanopropyl bonded-phase columns. After extraction, samples are separated by isocratic reversed-phase high-performance liquid chromatography. The parent drug, internal standard and a yet unidentified metabolite are detected by fluorescence. The method requires 1.0 ml of plasma or 0.1 ml of urine and has a lower limit of quantitation of 2 ng/ml with 10.9% relative standard deviation (R.S.D.). Method linearity has been established over a 2–800 ng/ml range when 1.0 ml of plasma is used. The intra- and inter-day imprecisions for the method are typically better than 6% and 11% R.S.D., respectively, in both plasma and urine over the entire dynamic range. The pooled estimate of bias is less than 5% and attests to the excellent accuracy.

INTRODUCTION

Nausea and vomiting are among the most common adverse effects of cancer chemotherapy and often contribute to patients' reluctance to undergo treatment. In practice, clinical oncologists aggressively advocate antiemetic therapy to prevent chemotherapy–induced nausea and vomiting in an attempt to improve patient psychological behavior and acceptance of treatment.

ADR-851 (hereafter referred to as I, Fig. 1), *S*(+)-4-amino-5-chloro-N-(2-pyrrolidinylmethyl)-2,3-dihydrobenzo[6]furan-7-carboxamide hydrochloride, is a member of a new class of compounds, synthesized at Adria Laboratories, with 5-hydroxytryptamine receptor blocking activity. It has shown promising results

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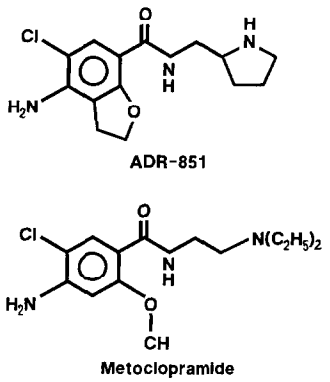

Fig. 1. Structures for I (ADR-851) and metoclopramide (I.S.).

in canine models of emesis against cisplatin- and dacarbazine-induced vomiting [1,2]. In order to elucidate the pharmacokinetic properties of I, bioanalytical methodology with sufficient sensitivity has been developed. Several bioanalytical methods for pharmacologically/chemically related compounds have appeared in the literature [3–6]. Our work describes a methodology for determining I in plasma and urine that employs a selective solid-phase extraction (SPE) technique, and high-performance liquid chromatography (HPLC) with fluorescence detection. The sample preparation procedure involves SPE of the compound and the internal standard from buffered samples (pH 7.0) using a cyanopropyl sorbent.

EXPERIMENTAL

Chemicals

Compound I was synthesized at Adria Laboratories. Metoclopramide · HCl (Sigma, St. Louis, MO, U.S.A.), trifluoroacetic acid (TFA) (EM Science, Gibbstown, NJ, U.S.A.), concentrated phosphoric acid and anhydrous dibasic potassium phosphate (Baker, Phillipsburg, NJ, U.S.A.) were all reagent grade. Triethylamine (TEA) (Gold Label) was obtained from Sigma. Acetonitrile was chromatography grade (EM Science). All reagents were used without further purification. Water was purified in-house, using a Milli-Q® water purification system (Millipore, Milford, MA, U.S.A.). Unless otherwise stated, all plasma standards and controls were prepared using human plasma.

Apparatus

The SPE columns contained 500 mg of unendcapped cyanopropyl/silica sorbent (p/n 624303, Analytichem, Harbor City, CA, U.S.A.), and were eluted on a vacuum manifold system from Burdick and Jackson (Muskegon, MI, U.S.A.). The chromatography column was Ultrasphere® ODS (5 μm; 25 cm × 4.6 mm I.D., Beckman, San Ramon, CA, U.S.A.). The guard column employed was an

RP-18 New Guard® (3 cm × 3.2 mm I.D., Brownlee, Santa, Clara, CA, U.S.A.). The chromatograph consisted of a pump (Model 400, ABI, Ramsey, NJ, U.S.A.), an autosampler (Model 9090, Varian, Walnut Creek, CA, U.S.A.), a 200-μl loop and a fluorescence detector (Model 980, ABI) attached to a data acquisition system (Nelson, Cupertino, CA, U.S.A.) with ACCESS*CHROM® software. The fluorescence detector (5-μl flow cell) employed a deuterium lamp and had an excitation wavelength (λ_{ex}) of 227 nm and a 345-nm cutoff emission filter. The photomultiplier current was set at 0.1 μA. The detector time constant was 2 s. A 1-V signal was sent from the detector to the data system. Small-volume (300 μl) polypropylene autosampler vials (p/n 1106A, Sunbrokers, Wilmington, NC, U.S.A.) were used to accommodate the reconstituted sample following the extraction. Sample dry down was performed with a vortex evaporator (Buchler, Fort Lee, NJ, U.S.A.).

HPLC conditions

The mobile phase consisted of 0.02 *M* phosphate buffer–acetonitrile–TEA (85:15:0.1, v/v). The phosphate buffer and the TEA were combined first, adjusted to pH 4.0 with concentrated phosphoric acid, to which acetonitrile was added. Isocratic separation, with a flow-rate of 1.0 ml/min, gave a typical back-pressure of 90 bars.

Preparation of standards

Stock solutions of I and metoclopramide were prepared at 100 μg/ml (as free bases). Plasma standards of I were prepared by ten-fold dilution of diluted solution standards with human plasma to yield concentrations of 800, 600, 200, 96.0, 48.0, 16.0, 9.60 and 3.84 ng/ml.

For quantification of I in urine, 100 μl of blank urine were added to 10 ml of solution standard before bringing to volume with 100 ml water and standards of 800, 400, 200, 100, 50.0 and 20.0 ng/ml were obtained.

Extraction procedure

An aliquot of plasma or standard sample (0.1–1.0 ml) was buffered with 1.0 ml of 0.02 *M* phosphate buffer (pH 7.0) and spiked with 100 μl of the internal standard (I.S.) solution (6 μg/ml). The mixture was briefly vortexed (5 s) and loaded onto a prewashed (2 × 2 ml of acetonitrile and 2 × 2 ml of water) cyanopropyl SPE column. The mixture on the loaded column was washed with water (2 × 2 ml) and acetonitrile (2 × 2 ml), and eluted with 1.0 ml of 2% TFA–acetonitrile in a disposable glass test tube. The eluent was then evaporated to dryness at 40°C and 90 kPa of vacuum. The residue was reconstituted with 250 μl of mobile phase, vortexed, transferred to an autosampler vial and injected into the chromatographic system. To obtain peak heights bracketed by the standard samples, the injection volume from the plasma extracts was varied between 5 and 90 μl. All standards and quality control samples required 90 μl per injection.

Urine samples were first diluted 1000-fold with water. A 1-ml aliquot of urine working standard or diluted urine sample was buffered, spiked and extracted as described above. All processed urine samples and standards required a 90-μl injection.

Calculations

Peak heights for the parent, metabolite and the I.S. were estimated with the ACCESS*CHROM software. Peak-height ratios of I to I.S. were calculated for all samples, standards and controls. Calibration curves of peak-height ratios *versus* nominal concentrations (ng/ml) were constructed to estimate linear regression parameters. A weight of 1 over the square of the standard deviation ($1/S.D.^2$) was assigned to each datum in the regression.

RESULTS AND DISCUSSION

Several analogues of I were evaluated but metoclopramide, a structurally related compound (Fig. 1), was selected as the I.S. for this method, primarily because it is readily available, stable under the assay conditions, demonstrates good recovery from the matrix and displays appropriate chromatographic properties (k' of 7 relative to 4 for I).

Chromatographic performance

The UV absorbance spectrum for I in mobile phase shows absorbance maxima at 227 and 277 nm. Although the possibility of interferences is less at 277 nm, the shorter excitation wavelength was chosen because of higher fluorescence quantum efficiency, which in turn could offer a lower detection limit.

Fig. 2a shows a chromatogram for a representative blank plasma obtained from the rat. No endogenous plasma constituents coelute with either I or the I.S., and the chromatographic windows are clean. One or two very small peaks occasionally eluted between the parent and I.S. peaks, but baseline resolution from the analytes did not cause any interference. Similar observations could also be made from the blank urine from the rat. The blanks obtained from the dog and man showed no interference either. Fig. 2b shows the presence of a possible metabolite in the 8-h rat plasma sample after intravenous dosing (10 mg/kg), apparently well resolved from the parent and the I.S. peaks (resolution factors: 4.8–12). No interfering peaks were apparent. The retention times for I and metoclopramide were 10.3 and 15.9 min, respectively. The peaks are well resolved ($R_s > 5$) and have good peak shape (peak asymmetry of 1.3).

Linearity

Correlation coefficients (r) obtained from the regression of five plasma calibration curves run on five separate days over a two-week period ranged from 0.998 to 1.000. Furthermore, the average bias associated with the back-calculated

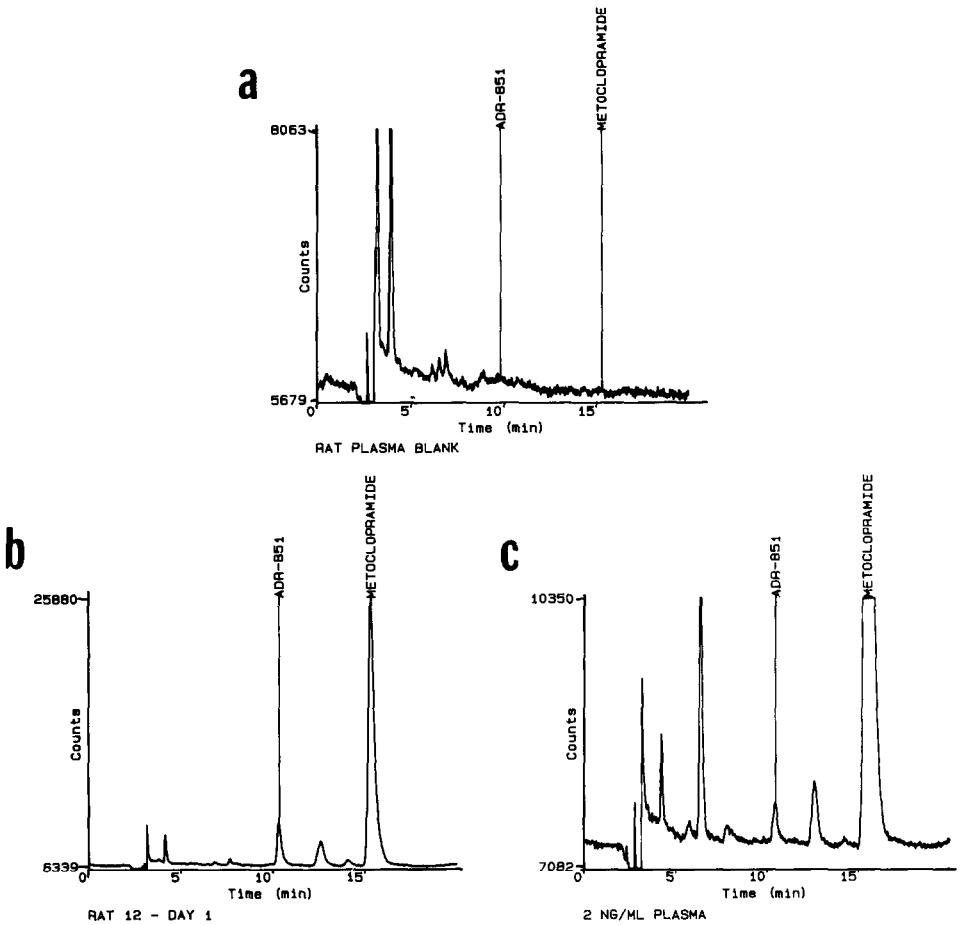

Fig. 2. (a) Typical chromatogram for blank plasma from rat. (b) Chromatogram of rat plasma sample collected 8 h after a 10 mg/kg intravenous dose of I [order of elution: I (parent) at 10.3 min, unidentified metabolite at 12.8 min and the I.S. at 15.9 min]. (c) Chromatogram of an extracted plasma standard of I at LLQ (2 ng/ml).

concentrations ranged from 0.3 to 5.0% usually, and was typically less than 2%, suggesting good agreement with the linear model over the entire calibration range.

With correlation coefficients ranging from 0.998 to 1.000, the linearity of the urine calibration curves appears to be excellent over a range of 20–800 ng/ml. The average bias associated with the back-calculated standard concentrations ranged from 1.0 to 5.2% with typical values of about 3.5%.

Precision

The precision estimates of replicate determinations for I in plasma, evaluated at four levels on each of five days, are summarized in Table I. The data shows that

TABLE I

ACCURACY AND PRECISION ESTIMATES FOR REPLICATE DETERMINATIONS OF I IN PLASMA

Label concentration of control sample (ng/ml)	Day	*n*	Mean concentration found (ng/ml)	Bias (%)	S.D. (ng/ml)	R.S.D. (%)		Pooled bias (inter-day) (%)
						Intra-day	Inter-day (pooled)	
3.84	1	5	4.23	+10.2	0.31	7.3		
	2	5	3.70	− 3.5	0.12	3.2		
	3	5	3.07	−20.0	0.15	4.9		
	4	5	3.56	− 7.4	0.08	2.2		
	5	5	3.72	− 3.0	0.13	3.5	11.2	−4.8
16.0	1	5	17.0	6.4	0.6	3.8		
	2	5	16.5	3.4	0.4	2.5		
	3	5	16.2	1.2	0.7	4.4		
	4	5	17.3	8.0	1.5	8.4		
	5	5	16.4	2.5	0.2	1.2	5.0	4.3
96.0	1	5	91.7	− 4.5	2.0	2.1		
	2	5	93.6	− 2.5	1.1	1.2		
	3	3	91.4	− 4.8	1.3	1.4		
	4	5	93.2	− 2.9	1.2	1.3		
	5	0	−	−	−	−	1.8	−3.5
600	1	5	539	−10.2	12	2.2		
	2	5	581	− 3.1	20	3.4		
	3	5	637	6.2	78	12.2		
	4	5	551	− 8.2	46	8.4		
	5	5	561	− 6.5	26	4.5	9.2	−4.4

the relative intraday imprecision ranged from 1.2 to 12.2% relative standard deviation (R.S.D.), with an average imprecision of 4.1% R.S.D. For plasma spikes, inter-day imprecision ranged from 1.8 to 11.2% R.S.D., with an average of 6.8% R.S.D. As shown in Table II, the intra-day imprecision for urine controls ranged from 4.3 to 7.9% R.S.D. at 20 ng/ml and from 2.3 to 4.4% R.S.D. at 400 ng/ml. The interday imprecision at these levels are 6.5 and 5.5% R.S.D., respectively. These values are consistent with the precision estimates obtained in plasma and attest to the excellent reproducibility of the assay.

Accuracy

Table I contains mean values for replicate determinations of I in plasma. Five replicates were prepared on each of the five days, except for the 96.0 ng/ml control which was replicated on only four days. The average bias was between 2 and 5% for all concentrations. The 95% confidence intervals of the mean concentrations always enclosed the true nominal spiked concentrations. Average biases for urine controls at 20 and 400 ng/ml were −4.9% and −2.0%, respectively. As with plasma, the biases are not significant because the nominal concentrations are within the 95% confidence intervals of the mean determined values.

Intrumental lower limit of detection (ILLD)

The ILLD was obtained by estimating the smallest amount of I which could be detected in the absence of matrix with a signal-to-noise ratio of approximately 3 [7]. For a 90-μl injection, 1 ng/ml is readily detectable, while 0.5 ng/ml is below the ILLD. An ILLD of 1 ng/ml corresponds to 90 pg of I on column.

Lower limit of quantitation (LLQ)

Replicate determinations of spiked plasma controls of I (0.960 ng/ml) resulted in an unacceptable 46% R.S.D. More precise estimates of concentration were obtained at 2.0 ng/ml, where the imprecision was an acceptable 10.9% R.S.D. [7]. A representative chromatogram for a 2.0 ng/ml control is shown in Fig. 2c.

Extraction efficiency

The mean (± S.D.) extraction efficiency was 87.7 ± 5.7% over 9.6–800 ng/ml for I and 88.4 ± 3.7% for metoclopramide at a spike of 600 ng/ml. There were no statistically significant differences ($p > 0.05$) in the recovery of I at three levels (9.6, 96 and 800 ng/ml) or of metoclopramide. Recovery, therefore, appears to be independent of concentration. Extraction efficiency of I from urine showed no concentration dependence and an average recovery of 85.6 ± 3.4% at levels of 20, 100 and 800 ng/ml. The recovery for metoclopramide from urine (80.0 ± 4.1%) was relatively similar to that from plasma.

Stability

The results of stability experiments at pH 3.0, 7.4 and 10 suggest no degrada-

TABLE II

ACCURACY AND PRECISION ESTIMATES FOR REPLICATE DETERMINATIONS OF I IN URINE

Label concentration of control sample (ng/ml)	Day	*n*	Mean concentration found (ng/ml)	Bias (%)	S.D. (ng/ml)	R.S.D. (%)		Pooled bias (inter-day) (%)
						Intra-day	Inter-day (pooled)	
20	1	5	18.8	−6.0	1.2	6.2		
	2	5	19.8	−1.0	0.9	4.3		
	3	5	18.5	−7.5	1.5	7.9	6.5	−5.0
400	1	5	400.7	0.2	9.1	2.3		
	2	5	368.3	−7.9	11.6	3.1		
	3	5	406.2	1.8	17.8	4.4	5.5	−2.0

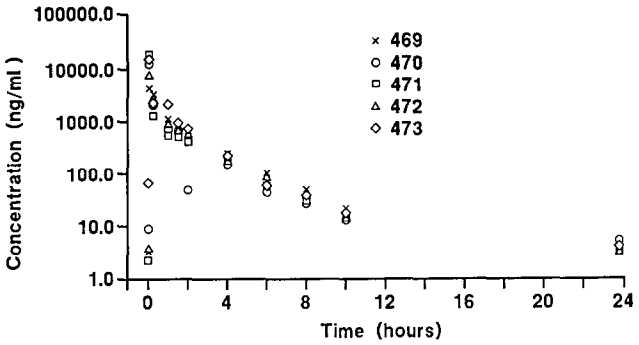

Fig. 3. Steady-state disposition profile of I in beagle dog plasma following a daily intravenous administration of 8 mg/kg I for 28 days.

tion of I up to 20.5 h at ambient temperature and light in aqueous solutions. Extracted plasma and urine samples, when reconstituted and stored in polypropylene autosampler vials, at room temperature and under ambient light conditions, showed no degradation for at least 100 and 24 h, respectively. Plasma controls at −20°C over a thirteen-week period showed no apparent degradation of I when cycled four times through freeze–thaw cycles.

Disposition profile

Fig. 3 shows the disposition profile of I in dogs following daily intravenous doses of 8 mg/kg for 28 days. The decline in plasma concentrations as a function of time appears to be multiphasic and the terminal phase shows as estimated half-life of 6.4 h. The plasma concentration range of I was 0.003–18 μg/ml. A detailed description of pharmacokinetics of I will be presented separately in a subsequent paper.

CONCLUSION

We have described a sensitive (2 ng/ml), specific, precise (<12% R.S.D. at LLQ) and accurate (<10% bias) method for the quantitation of I in plasma and urine. We are currently using this method for supporting the Phase I clinical pharmacology program of I as an inhibitor of chemotherapy-induced nausea and vomiting.

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