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Quantitation of loperamide and *N*-demethyl-loperamide in human plasma using electrospray ionization with selected reaction ion monitoring liquid chromatography–mass spectrometry

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Abstract

We report here the development and validation of an LC–MS method for quantitation of loperamide (LOP) and its *N*-demethyl metabolite (DMLOP) in human plasma. *O*-Acetyl-loperamide (A-LOP) was synthesized by us for use as an internal standard in the assay. After addition of the internal standard, the compounds of interest were extracted with methyl *tert*.-butylether and separated by HPLC on a C_{18} reversed-phase column using an acetonitrile–water gradient containing 20 m*M* ammonium acetate. The three compounds were well separated by HPLC and no interfering peaks were detected at the usual concentrations found in plasma. Analytes were quantitated using positive electrospray ionization in a triple quadrupole mass spectrometer operating in the MS–MS mode. Selected reaction monitoring was used to quantify LOP (*m*/*z* 477→266), DMLOP (*m*/*z* 463→252) and A-LOP (*m*/*z* 519→266) on ions formed by loss of the 4-(*p*-chlorophenyl)-4-hydroxy-piperidyl group upon low energy collision-induced dissociation. Calibration curves, which were linear over the range 1.04 to 41.7 pmol/ml (LOP) and 1.55 to 41.9 pmol/ml (DMLOP), were run contemporaneously with each batch of samples, along with low (4.2 pmol/ml), medium (16.7 pmol/ml) and high (33.4 pmol/ml) quality control samples. The lower limit of quantitation (LLQ) of LOP and DMLOP was about 0.25 pmol/ml in plasma. The extraction efficiency of LOP and DMLOP from human plasma was 72.3±1.50% (range: 70.7–73.7%) and 79.4±12.8% (64.9–88.8%), respectively. The intra- and inter-assay variability of LOP and DMLOP ranged from 2.1 to 14.5% for the low, medium and high quality control samples. The method has been used successfully to study loperamide pharmacokinetics in adult humans.

Keywords: Loperamide; *N*-Demethyl-loperamide

1. Introduction

Loperamide (1), 4-(*p*-chlorophenyl)-4-hydroxy-*N*,*N*,-dimethyl-α,α-diphenyl-1-piperidine butyramide hydrochloride (LOP), is a moderately potent opiate

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agonist used to treat diarrhea by virtue of its selective action in the gastrointestinal tract, but not on the central nervous system. Its major metabolite, *N*-demethyl-loperamide (2), 4-(*p*-chlorophenyl)-4-hydroxy-*N*-methyl-α,α-diphenyl-1-piperidine butyramide hydrochloride (DMLOP), is not active against diarrhea [1] nor does it bind to opiate receptors.

The pharmacokinetics of loperamide in human have been described [2–6]. Several radioimmunoassays (RIAs) for determination of loperamide in serum and urine have been reported [3–5]. Although the RIA methods were sensitive, they lacked adequate selectivity for pharmacokinetic and drug metabolism studies. Several high-performance liquid chromatography (HPLC) methods have been described which report the separation of loperamide from its metabolites and other compounds [6–11]. A gas chromatography–mass spectrometry (GC–MS) method has also been described by Leis and Gleispach [11]. The detection limit (1 ng/ml) reported in these studies was not adequate to monitor therapeutic levels of LOP and DMLOP in plasma at the lower limits we anticipated. The use of a UV detector at 210 nm was unable to achieve the sensitivity we required because of the low molar absorptivity of the UV chromophores in loperamide and the presence of UV absorbing interference in plasma [6–8]. Although the HPLC methods, when used with a radioactivity detector [6], afforded adequate sensitivity and specificity, the use of radiolabeled drugs is difficult to justify for routine clinical pharmacokinetic studies. Hewala has reported an improved assay using an electrochemical detector [10] in which the limit of quantification was 0.4 pmol/ml. However, the chromatograms showed a number of interfering peaks in plasma samples that limited its utility.

To further investigate the mechanism of reverse cellular transport of LOP and DMLOP by P-glycoprotein drug transporters, we required a sensitive, reliable and rapid analytical method. In this study, we anticipated that the lower concentration of LOP and DMLOP in human plasma would be 0.5–8 pmol/ml (0.2–4 ng/ml). Therefore, we developed a sensitive and specific liquid chromatographic–tandem mass spectrometric (LC–MS–MS) assay using electrospray ionization and selective reaction monitoring (SRM) to determine LOP and DMLOP in human plasma. As part of the assay we synthesized *O*-acetyl-loperamide (A-LOP) for use as an internal standard. This method has been used successfully to study loperamide pharmacokinetics in adult humans.

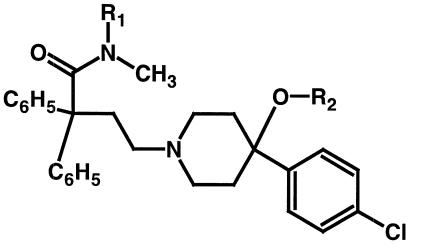
2. Experimental

2.1. Chemicals

Loperamide hydrochloride and methyl *tert*.-butylether (MTBE) (HPLC grade) were purchased from Sigma (St. Louis, MO, USA). *N*-Demethyl-loperamide was a gift from (Janssen Pharmaceutica, Beerse, Belgium). Acetonitrile (UV grade) was obtained from EM Science (Gibbstown, NJ, USA). *O*-Acetyl-loperamide was synthesized by acetylation of loperamide with acetic anhydride–pyridine (1:3) at 80°C for 5 h. The product was purified by column chromatography on silica and crystallized upon evaporation of the eluting solvent in 76% yield. The purity of the final product was 99.8% as determined by LC–MS using the chromatographic system described below. The chemical structures (see Scheme 1) and molecular masses were confirmed by nuclear magnetic resonance (NMR) and MS.

2.2. Instrumentation and chromatographic conditions

Chromatographic separations were performed using a Waters 2690 integrated HPLC system inter-



Loperamide	$R_1 = CH_3$	$R_2 = H$
N-Demethyl-Loperamide	$R_1 = H$	$R_2 = H$
O-Acetyl-Loperamide	$R_1 = CH_3$	$R_2 = CH_3CO$

Scheme 1. Structures of loperamide, *N*-demethyl-loperamide, and *O*-acetyl-loperamide.

faced to the mass spectrometer (Waters, Milford, MA, USA). Analytical separations were performed on a 150×2.1 mm column packed with 5 μm Zorbax XDB-C_{18} reversed-phase silica (MAC-MOD Analytical, Chadds Ford, PA, USA). Compounds were eluted using a binary gradient solvent system consisting of 20 m*M* ammonium acetate (pH 6.6) in water (solvent A) and acetonitrile (solvent B). The gradient profile consisted of an initial isocratic hold at 40% B (0–1.5 min), followed by a linear gradient increasing to 80% B (1.5–8 min), and held at 80% B (8–9 min). The gradient was reversed from 80% B to 40% B (9–12 min) to reestablish the initial conditions resulting in a cycle time of 12 min between injections. The peaks of interest eluted with a retention time of 5–7 min at a flow-rate of 0.3 ml/min using this gradient program. Mass spectra were obtained in positive ion mode on a Finnigan TSQ 7000 triple quadrupole (Finnigan, San Jose, CA, USA) equipped with an electrospray and an atmospheric pressure chemical ionization source.

2.3. Mass spectrometry

Standard solutions (10 nmol/ml) were introduced by syringe infusion pump directly into the mass spectrometer at 5 μl/min. Positive ion mass spectra of LOP, DMLOP and A-LOP were obtained by scanning quadrupole 1 (Q1) from *m/z* 100–550 at 1 scan/s at unit resolution. The electrospray voltage was 4.00 kV and the heated capillary lens was run at 200°C to facilitate mobile phase desolvation. The tube lens in the interface was set at 65 V to minimize fragmentation in the ion source and give optimum yield of parent ions. For tandem MS experiments, the collision gas (Ar) pressure was set to 2.5 mTorr (1 Torr=133.322 Pa). Product ion spectra were obtained by scanning Q3 as just described for Q1. The fragmentation pattern was compared at collision energies of −15, −25, −35 and −45 eV using a laboratory frame of reference in order to determine the optimal collision energy. The spectra reported here were averaged for 1 min. SRM was used to quantify loperamide (*m/z* 477→266), DMLOP (*m/z* 463→252) and A-LOP (*m/z* 519→266) using a collision energy of −35 eV (laboratory frame of reference). The ion cycle time was 500 ms, which resulted in a dwell time of 167 ms per mass transition.

2.4. Preparation of stock solutions, calibration standards and quality control samples

Primary stock solutions of loperamide hydrochloride (0.26 μmol/ml), *N*-demethyl-loperamide (0.39 μmol/ml) and *O*-acetyl-loperamide (0.31 μmol/ml) were prepared in methanol. Calibration standards and quality control samples were prepared from separately weighed primary stock solutions. All stock solutions were stored at 4°C when not in use.

Calibration standards of LOP and DMLOP were prepared by spiking an appropriate amount of the concentrated stock solutions in control plasma obtained from healthy, non-smoking volunteers who were not drinking coffee. Calibration curves covered the range 1.0–41.7 pmol/ml and 1.5–61.9 pmol/ml, respectively. Quality control (QC) samples were prepared in blank control plasma at concentrations of 4.14, 16.7 and 33.4 pmol/ml for LOP and 6.19, 24.8 and 49.5 pmol/ml for DMLOP.

2.5. Sample preparation and extraction procedures

A 10-μl aliquot of the *O*-acetyl-loperamide (788.9 pmol/ml) internal standard was added to 0.5 ml of each plasma sample and vortex-mixed. The plasma was made alkaline by addition of 1 ml of 10% sodium carbonate buffer (pH 9.6), the target compounds were extracted with of 8 ml MTBE for 10 min, and centrifuged at 700 *g* for 10 min. The organic layer was transferred to another tube and evaporated under N_2 in a water bath at 40°C. The residue was reconstituted in 200 μl of 20 m*M* ammonium acetate–methanol (1:4) and filtered through a 0.22-μm nylon syringe filter (Corning, Corning, NY, USA). A 40-μl aliquot of the reconstituted sample was injected onto the analytical column.

2.6. Assay validation

2.6.1. Linearity

Calibration standards of six concentrations of LOP ranged 1.04–41.7 pmol/ml and DMLOP ranged 1.55–61.89 pmol/ml were extracted and assayed. A

least-squares linear regression model was used to determine the plasma concentration from the peak height ratio data.

2.6.2. Precision and accuracy

The precision of the assay was determined from the low, medium and high QC plasma samples by replicate analyses of three concentrations of LOP (4.2, 16.7, 33.4 pmol/ml) and DMLOP (6.2, 24.8, 49.5 pmol/ml). Intra-day precision was determined by repeated analysis of the group of standards on one day ($n=5$), and inter-day precision and accuracy was determined by repeated analysis on five consecutive days ($n=1$ series per day). The concentration of each sample was determined using calibration standards prepared on the same day. Accuracy is defined as the relative deviation in the computed value (E) of a standard from that of its true value (T) expressed as a percentage (RE %). It was calculated using the following formula RE $\%=(E-T)/T\cdot 100$. Assay precision was defined as the relative standard deviation (SD) from the mean (M), calculated using the equation RSD $\%=\mathrm{SD}/M\cdot 100$.

2.6.3. Extraction and recovery

The absolute recovery (extraction efficiency) of LOP and DMLOP through the extraction procedures was determined at low, medium and high concentrations by the external standard method. A known amount of LOP and DMLOP was added to human plasma prior to extraction as described in Section 2.5. The internal standard (A-LOP) was added after extraction to eliminate bias introduced by sample processing. Concentration of the two compounds was calculated using the calibration curves prepared on the same day.

2.6.4. Assay specificity

Specificity was assessed by extracting samples of five different batches of blank plasma, and comparing these plasma with LOP (1.04 pmol/ml) and DMLOP (1.55 pmol/ml) that were the lowest concentration of LOP and DMLOP in calibration standard. The chromatograms were also visually inspected for interfering chromatographic peaks from endogenous substances. Plasma samples containing a co-administered medication, quinidine, was also checked.

3. Results and discussion

LOP, DMLOP and A-LOP are nonpolar, hydrophobic compounds with similar molecular structures. As a consequence they exhibited similar extraction efficiency and chromatographic behavior. Moreover, they had similar mass spectral ionization properties and fragmentation patterns which facilitated their analysis by selected reaction monitoring techniques.

3.1. Chromatography

The selection of mobile phase components was a critical factor in achieving good chromatographic peak shape and resolution. Ammonium acetate was selected as a buffer and methanol and acetonitrile were evaluated as mobile phases. The ionization efficiency of LOP and DMLOP was about the same in either solvents. However, acetonitrile exhibited better selectivity than methanol with regard to optimizing the separation of the target compounds. A-LOP was selected as an internal standard because its molecular mass, chemical properties and mass spectral fragmentation were similar to those of LOP and DMLOP. Preliminary studies with other internal standards, such as cyclizine [8], were unsatisfactory. The reproducibility and linearity of the calibration curves based on cyclizine exhibited too much variability in our hands to make it a suitable internal standard. Moreover, A-LOP was easy and inexpensive to prepare and purify. In order to obtain good separation of the target compounds and to achieve as short a run time as practical, a gradient elution system was developed. Representative chromatograms are shown in Fig. 1 in which the retention times were 5.55 min (LOP), 5.00 min (DMLOP) and 6.48 min (A-LOP). Care was taken to adjust the solvent composition such that the target compounds were separated from the column void volume under which eluted non-specific interfering compounds that were co-extracted from plasma and which tended to suppress ionization.

3.2. Optimization of MS conditions

To our knowledge, a tandem LC–MS method for quantitation of LOP and DMLOP in human plasma has not been reported. Both atmospheric pressure

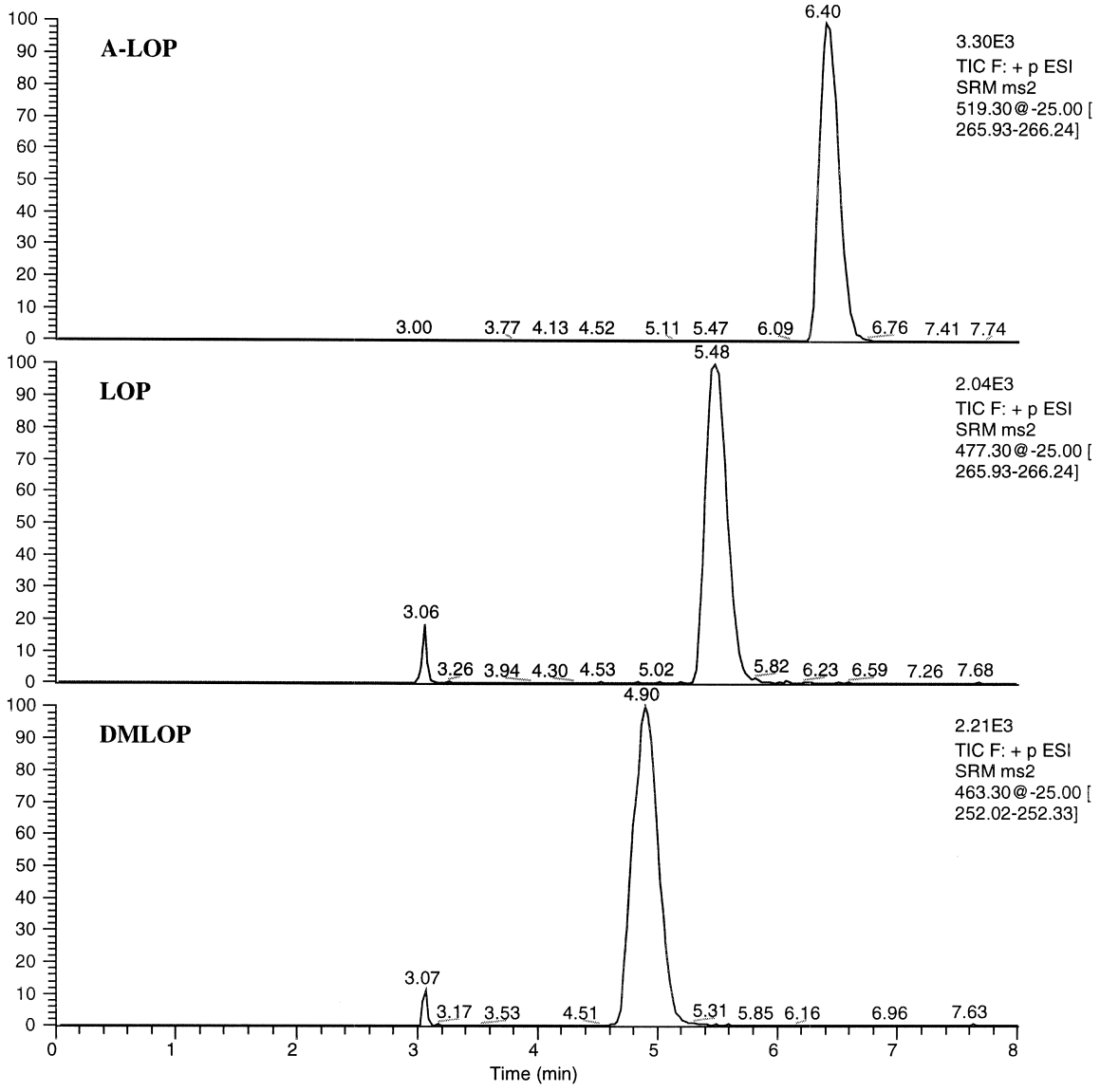


Fig. 1. Typical chromatograms of (A) plasma spiked with LOP (4.2 pmol/ml) and DMLOP (6.2 pmol/ml); (B) blank plasma; and (C) plasma obtained from a volunteer 4.5 h after loperamide administration. Each sample was spiked with A-LOP (78.8 pmol/ml), extracted as described in the text and a 40-μl aliquot (of 200 μl total) was analyzed by tandem LC–MS.

chemical ionization (APCI) and electrospray ionization (ESI) sources were evaluated for assay development in positive ion mode. In general, ESI produced greater sensitivity and exhibited less interference than we were able to achieve with APCI, thus ESI was selected for this assay.

ESI produced abundant protonated molecular ions ([M+H]) for LOP $m/z=477$, DMLOP $m/z=463$ and A-LOP, $m/z=519$ with little or no fragmentation at low tube lens voltages. In order to minimize undesirable fragmentation at the *N*-phenylpiperidine ring due to in-source collision-induced dissociation (CID), the tube lens voltage was set to <75 V. Product ion mass spectra were recorded at collision

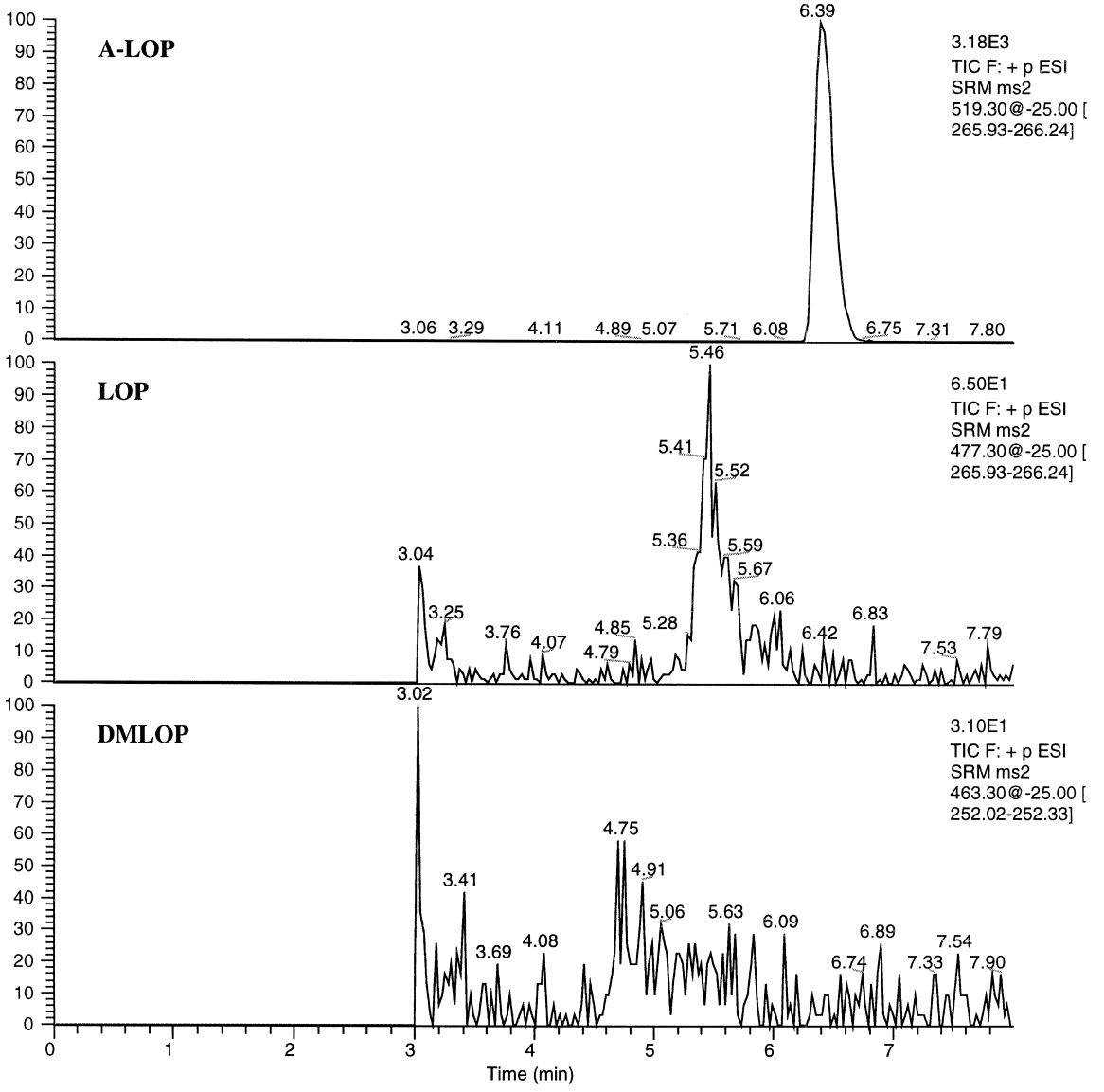


Fig. 1. (*continued*)

energies of −15, −25, −35 and −45 eV. Collision energies of −25 to −35 eV produced good CID spectra with efficient production of a single product ion for LOP (*m*/*z* 477→266), A-LOP (*m*/*z* 519→266), and DMLOP (*m*/*z* 463→253). For convenience a single collision energy of −35 eV was chosen for MS–MS quantitation.

3.3. Linearity and lower limit of quantitation

The calibration curves which relate the concentration of LOP or DMLOP to the peak height ratio of LOP (or DMLOP) to internal standard were linear over the range of 1.0 to 41.7 pmol/ml (LOP) and 1.6 to 61.9 pmol/ml (DMLOP). A typical calibration

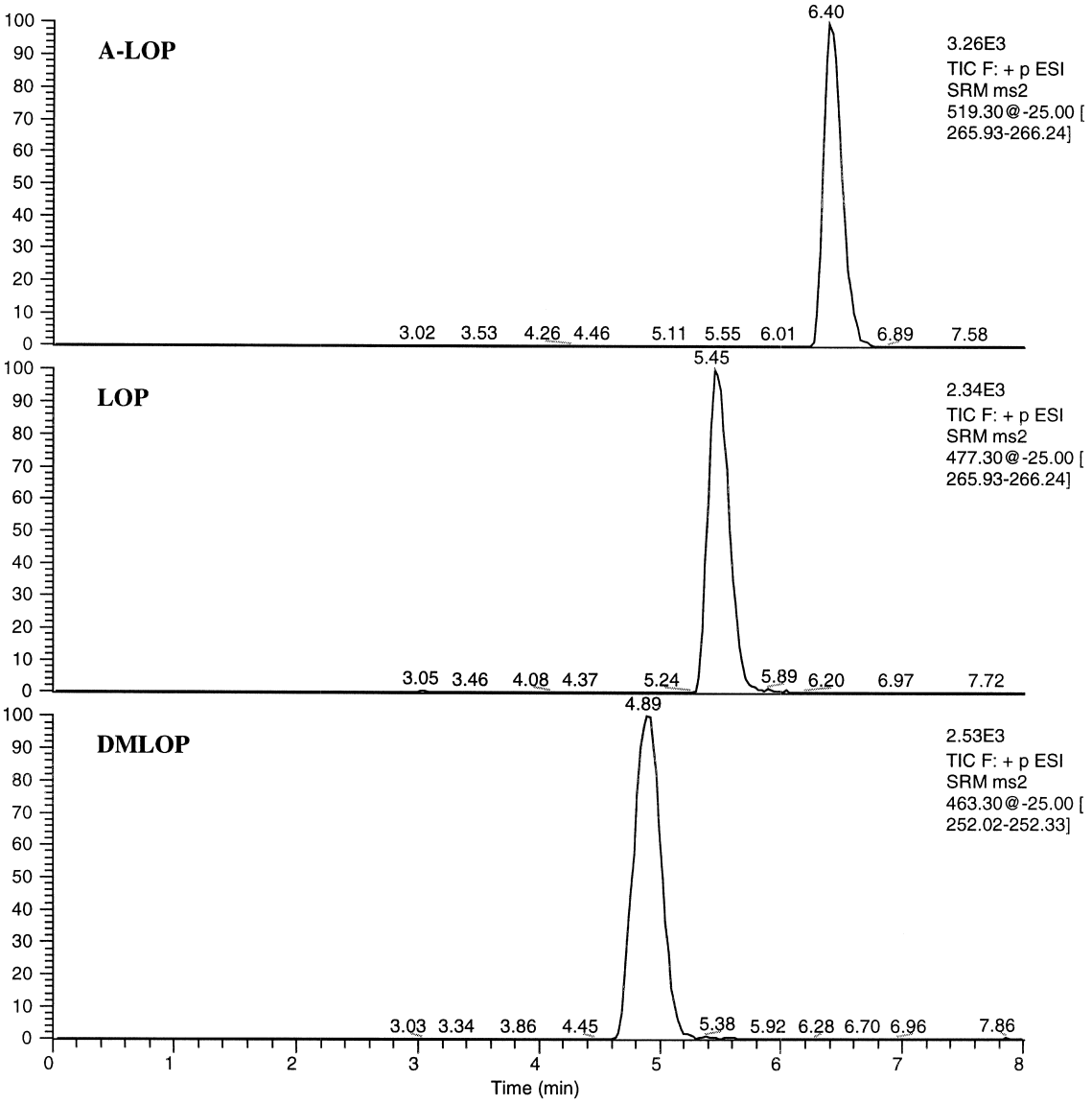


Fig. 1. (*continued*)

curve for LOP had a slope of 0.1198, an intercept of 0.0556 and R^2=0.9989, while those of DMLOP were 0.0574, 0.0645 and 0.9980, respectively. A calibration curve was prepared contemporaneously with each batch of samples. The absolute detection limits of LOP and DMLOP were 5.2 fmol and 7.8 fmol, respectively ($S/N>3$). The lower limit of quantitation (LLQ) of the assay, defined at a $S/N=$ 10:1, was 0.25 pmol/ml for both compounds. The lowest calibration points were 4- to 6-times greater than the LLQ. The dynamic range of the assay was arbitrarily restricted to cover only the plasma concentration range anticipated for the 24 h pharmacokinetic studies. The linear dynamic range of the

Table 1
Precision and accuracy of the assay for determination of LOP and DMLOP in plasma ($n=5$)

Compound	Added to plasma (pmol/ml)	Intra-assay			Inter-assay		
		Measured concentration (pmol/ml) (mean±SD)	RE (%)	RSD (%)	Measured concentration (pmol/ml) (mean±SD)	RE (%)	RSD (%)
LOP	4.17	4.24±0.19	1.7	4.6	4.67±0.56	12.0	12.2
	16.69	16.48±0.35	−1.3	2.1	16.97±2.46	1.7	14.5
	33.38	33.24±1.95	−0.4	5.9	32.28±2.67	−3.3	8.3
DMLOP	6.19	5.19±0.35	−16.2	6.9	6.18±0.67	−0.2	10.9
	24.75	24.49±1.98	−1.1	8.1	22.74±1.67	−8.1	7.3
	49.51	48.13±4.67	−2.8	9.7	48.72±3.69	−1.6	7.6

instrument could easily accommodate a 500-fold range of concentration in plasma.

3.4. Precision and accuracy

The intra- and inter-day ($n=5$) precision and accuracy, shown in Table 1, were satisfactory for our purposes. The intra-day relative standard deviation (RSD) for LOP was 2.1–5.9%, while that for DMLOP was 6.9–9.7% for the three QC standards. The inter-day RSD for LOP was 8.3–14.5%, while that for DMLOP was 7.3–10.9% for the three QC standards.

3.5. Extraction recovery

The mean recovery of LOP and DMLOP from human plasma was 72.3±1.50% (range: 70.7–73.7%) for LOP and 79.4±12.8% (64.9–88.8%) for DMLOP, respectively. The recovery data reported here is the average for the three QC standards shown in Table 2.

3.6. Specificity

Despite the relatively simple liquid–liquid extraction used in this procedure, a high degree of specificity was achieved by tandem mass spectrometry in the selective reaction ion monitoring mode. Typical plasma extracts showed little or no interference (Fig. 1) in the retention time range of the target compounds. Occasionally a small background peak was seen in blank plasma in the LOP and DMLOP SRM traces. But most often no additional peaks were found, even in plasma samples from subjects who received other drugs such as quinidine. However, we should note that there were non-specific interferences that eluted in the column void volume that necessitated diverting the eluent to waste for the first 3 min, as shown in Fig. 1B.

3.7. Application

The method has been used to simultaneously define plasma concentration vs. time profiles for loperamide and its metabolite *N*-demethyl-

Table 2
Extraction efficiency of LOP and DMLOP from plasma ($n=5$)

Compound	Added concentration (pmol/ml)	Measured concentration (pmol/ml) (mean±SD)	Extraction recovery (%)	RSD (%)
LOP	4.17	3.02±0.19	72.4	6.2
	16.69	12.30±1.61	73.7	13.1
	33.38	23.59±0.96	70.7	4.1
DMLOP	6.19	4.02±0.13	64.9	3.6
	24.76	21.99±1.76	88.8	8.0
	49.51	41.90±2.49	84.6	5.9

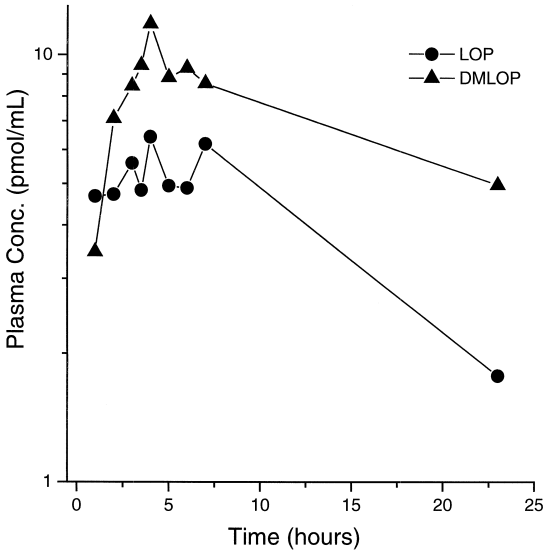


Fig. 2. Concentration vs. time profiles of LOP and DMLOP in plasma.

loperamide in subjects following a single oral bolus of 16 mg loperamide hydrochloride. Fig. 2 shows an example of plasma pharmacokinetics in one of the subjects. In this protocol, LOP was given orally to a subject and plasma was collected at frequent intervals for 7 h, with a final point collected at 24 h.

4. Conclusion

In the last 15 years tandem mass spectrometry has repeatedly proven to be a powerful technique for rapid, quantitative determination of drugs and metabolites in physiologic fluids. This assay achieved higher sensitivity and better specificity than other methods for analysis of loperamide and its major *N*-demethyl metabolite in human plasma. The LLQ, 0.25 pmol/ml for both compounds, was better than attainable by HPLC–electrochemical detection (EC), HPLC–UV and GC–MS. The calibration curve was linear over a 50-fold range. Moreover, A-LOP proved to be a good internal standard for this assay. Because of the high selectivity attainable by SRM, no significant interference caused by endogenous compounds or co-administered medications were observed, thus allowing use of a simple liquid–liquid extraction. This simple, rapid and robust assay was successfully used to study pharmacokinetics of loperamide after a 16 mg oral dose.

Acknowledgements

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