Journal of Chromatography B, 895–896 (2012) 56–64

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

High-sensitivity liquid chromatography–tandem mass spectrometry for the simultaneous determination of five drugs and their cytochrome P450-specific probe metabolites in human plasma

Kyung-Suk Oh, Su-Jin Park, Dhananjay D. Shinde, Jae-Gook Shin**, Dong-Hyun Kim*

Department of Pharmacology and PharmacoGenomics Research Center, Inje University College of Medicine, Busan, Republic of Korea

ARTICLE INFO

Article history:
Received 11 January 2012
Accepted 12 March 2012
Available online 19 March 2012

Keywords:
LC–MS/MS
Cocktail dosing
CYP
Phenotyping
Plasma
Simultaneous analysis

ABSTRACT

A sensitive liquid chromatography–tandem mass spectrometric (LC–MS/MS) method with electrospray ionization was developed for the simultaneous quantitation of five probe drugs and their metabolites in human plasma for assessing the *in vivo* activities of cytochrome P450 (CYP). CYP isoform specific substrates and their metabolites of CYP1A2 (caffeine), CYP2C9 (losartan), CYP2C19 (omeprazole), CYP2D6 (dextromethorphan) and CYP3A (midazolam) were all simultaneously analyzed using LC–MS/MS after administration of a mixture of five drugs (*i.e.*, a "cocktail approach") to healthy volunteers. The assay uses propranolol as an internal standard; dual liquid extraction; a Xbridge MS C_{18} (100 mm × 2.1 mm, 3.5 μm) column; a gradient mobile phase of 0.1% formic acid/acetonitrile (7/3 → 3/7); mass spectrometric detection in positive ion mode. The method was validated from 5 to 500 ng/mL for caffeine and paraxanthine, 0.1–40 ng/mL for losartan and EXP3174, 0.05–20 ng/mL for omeprazole and 5-hydroxyomeprazole, 0.008–0.8 ng/mL for dextromethorphan and dextrorphan, 0.01–1.0 ng/mL for midazolam, and 0.04–4 ng/mL for 1′-hydroxymidazolam. The intra- and inter-day precision over the concentration ranges for all analytes were lower than 12.5% and 13.8% (relative standard deviation, %RSD), and accuracy was between 86.5% and 108.4% and between 87.0% and 107.0%, respectively. This highly sensitive and quantitative method allowed a pharmacokinetic study in subjects receiving doses 10–100 times lower than typical therapeutic doses.

1. Introduction

The cytochrome P450 (CYP) enzymes comprise a superfamily of hemoproteins and are responsible for the oxidative metabolism of various endogenous substrates and xenobiotics [1]. Among the CYP isoforms, families 1 through 4 are involved in the metabolism of drugs. More than 90%-marketed drugs are metabolized by the CYP1A2, 2D6, 2C9, 2C19 and 3A isoforms [2]. The activities of these CYP isoforms show large interindividual and/or ethnic differences [3]. The modulation of drug-metabolizing activities can cause various pharmacological or toxicological consequences. The inhibition of these enzymes increases plasma levels of the parent drug or decreases the formation of active metabolites. Conversely, enzyme induction can lead to decreased levels of the parent drug or may increase the formation of toxic intermediates. These factors can often result in increased incidence of unwanted side effects or therapeutic failure. Therefore, precise and reliable estimations of the *in vivo* activity of individual CYP isoforms are essential for the evaluation of drug–drug interactions (DDIs). Number of specific probe drugs have been characterized and used to determine the real-time, *in vivo* activity of various CYP isoforms, including midazolam for CYP3A and dextromethorphan for CYP2D6 [4].

In addition to individual assessments of CYP isoform activity, the simultaneous administration of multiple probe drugs (*i.e.*, a 'cocktail' approach) has become popular as a means of providing information on multiple CYP isoform activities in a single experiment [5]. Several cocktail methods, such as the Cooperstown 5+1 cocktail and the Karolinska cocktail, have been developed since the 'Pittsburgh cocktail' was first introduced in 1997 [6–9]. However, these approaches are somewhat limited because mephenytoin, debrisoquine, and tolbutamide are no longer commercially available in many parts of the world. Moreover, the data for flurbiprofen are not entirely clear and caffeine is more widespread than phenacetin [10]. In a previous report,

* Corresponding author at: Department of Pharmacology and PharmacoGenomics Research Center, Inje University College of Medicine, #633-165 Gaegum-Dong, Jin-Gu, Busan 614-735, Republic of Korea. Tel.: +82 51 890 6411; fax: +82 51 893 1232.
** Corresponding author at: Department of Pharmacology and PharmacoGenomics Research Center, Inje University College of Medicine, #633-165 Gaegum-Dong, Jin-Gu, Busan 614-735, Republic of Korea. Tel.: +82 51 890 6709; fax: +82 51 893 1232.
E-mail addresses: phshinjg@inje.ac.kr (J.-G. Shin), dhkim@inje.ac.kr (D.-H. Kim).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2012.03.014

we described the "Inje cocktail" consisting of caffeine, losartan, omeprazole, dextromethorphan, and midazolam, which act as probe drugs for CYP1A2, 2C9, 2C19, 2D6, and 3A4, respectively [9]. These probe drugs are commercially available; they have low adverse effect profiles and are specific for CYP isoforms and applicable to *in vivo* studies.

Recently, microdosing (maximum dose of 100 μg) has been introduced in clinical studies to minimize the risk of harmful events to human subjects [11]. This can also reduce the influence of low-affinity, high-capacity enzymes. For new drug development, microdose trials can be used to identify unsuitable pharmacokinetic properties at an early stage of development, thereby reducing cost and time [12]. To date, there is no report to estimate *in vivo* P450 isoform activities using low-dose probe drugs for DDIs. While microdosing is beneficial in terms of side effects and possible interactions, it requires extremely sensitive analytical tool that are able to detect and quantitate both the probe drugs and their metabolites. Although several investigators reported a fast gradient LC/MS method for the simultaneous determination of CYP substrates and their metabolites [13–15], those methods could not be applied to current minidose clinical study due to different CYP probe drug composition or low analytical sensitivities.

The present report describes a highly sensitive LC–MS/MS method with electrospray ionization (ESI) that was developed to simultaneously detect five CYP isoform-specific probe drugs and their metabolites in human plasma. In addition, the utility of this method was demonstrated by pharmacokinetic analyses of the probe drugs and their metabolites after the concomitant oral administration of low doses of caffeine (10 mg), losartan (2 mg), omeprazole (200 μg), dextromethorphan (2 mg), and midazolam (100 μg) to healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

Caffeine (purity ≥99%), paraxanthine (purity ≥98%), dextromethorphan hydrobromide monohydrate (purity ≥99%), dextrorphan (purity ≥99%), and propranolol hydrochloride (purity ≥99%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Midazolam (purity 98%), 1′-hydroxymidazolam (purity 98%), and EXP3174 (purity 98%) were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Omeprazole (purity 99.9%) was acquired from USP (Rockville, MD, USA). 5-Hydroxyomeprazole (purity 99.8%) was obtained Astra-Zeneca (Mölndal, Sweden). Losartan potassium (purity 98%) was purchased from Merck (Rahway, NJ, USA). HPLC-grade acetonitrile and methanol were obtained from Burdick & Jackson (Muskegon, MI, USA) and ethyl acetate was purchased from J.T. Baker (Phillipsburg, PA, USA). Acetic acid and formic acid were purchased from Sigma–Aldrich and purified water (18.2 Ω cm) was processed with a Milli-Q water system (Millipore, Molsheim, France) and used throughout the study.

2.2. Preparation of standards and quality control samples

Primary stock solutions of probe drugs, their metabolites, and propranolol were prepared at 1 mg/mL as free base in methanol. Working solutions of each analyte were prepared by diluting the stock solutions with methanol. Propranolol (100 ng/mL in methanol) was used as an internal standard (IS). All stock solutions were stored at −20 °C. Calibration curves were generated using six calibration standards. Blank plasma samples were prepared by adding working solutions to drug-free blank plasma to final concentrations of 5, 10, 25, 100, 250, and 500 ng/mL for caffeine and paraxanthine, 0.1, 0.2, 1, 2, 10, and 40 ng/mL for losartan and EXP3174, 0.05, 0.1, 0.5, 1, 5, and 20 ng/mL for omeprazole and 5-hydroxyomeprazole, 0.008, 0.016, 0.04, 0.16, 0.4, and 0.8 ng/mL for dextromethorphan and dextrorphan, 0.01, 0.02, 0.05, 0.2, 0.5, and 1 ng/mL for midazolam, and 0.04, 0.08, 0.2, 0.8, 2, and 4 ng/mL for 1′-hydroxymidazolam. Calibration curves for the analytes in human plasma were derived from their peak area ratios relative to that of the IS using linear regression with a weighting factor. Quality control (QC) samples were prepared at final concentrations of 5, 15, 60, and 300 ng/mL for caffeine and paraxanthine, 0.1, 0.3, 2.4, and 24 ng/mL for losartan and EXP3174, 0.05, 0.15, 1.2, and 12 ng/mL for omeprazole and 5-hydroxyomeprazole, 0.008, 0.024, 0.096, and 0.48 ng/mL for dextromethorphan and dextrorphan, 0.01, 0.03, 0.12, and 0.6 ng/mL for midazolam, and 0.04, 0.12, 0.48, and 2.4 ng/mL for 1′-hydroxymidazolam. All standards and QC samples were aliquoted into pre-labeled, 15-mL Corning glass tubes and stored frozen at −20 °C.

2.3. Sample preparation

A dual liquid–liquid extraction method was used to extract the analytes from human plasma. To an aliquot of 0.45 mL plasma, 50 μL of IS solution (100 ng/mL) was added and mixed for 10 s on a Vortex mixer (Scientific Industries, Bohemia, NY, USA). Three milliliters ethyl acetate was then added to the samples and vortexed for 10 min. The samples were then centrifuged at 3000 rpm for 10 min. The organic layer was separated and transferred to another tube. The aqueous layer was then acidified by adding 0.1 mL of 1 M acetic acid and extracted with 3 mL of ethyl acetate. The organic layers from all extractions were pooled and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 μL of methanol, and 5 μL of the sample was injected into the LC–MS/MS system. All of the prepared samples were kept in an autosampler at 4 °C until injection.

2.4. LC–MS/MS methods

An Agilent 1100 series (Agilent, Wilmington, DE, USA) LC system consisting of an autosampler, binary pump, and column oven was used for separating the samples. The separation was performed on an Xbridge MS C_{18} column (100 mm × 2.1 mm, i.d., 3.5 μm; Waters Corporation, Milford, MA, USA). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A gradient elution was used at a flow rate of 0.2 mL/min. The initial buffer composition was 30% B, which was maintained for 1 min then linearly changed to 70% B over 4 min. This was maintained for an additional 5 min and the column was re-equilibrated to its initial conditions for 1 min. The column was maintained at ambient temperature. The HPLC system was coupled to a QTrap 5500 triple quadrupole mass spectrometer (Applied Biosystems, Concord, ON, Canada) equipped with a turbo ion spray source.

Electrospray ionization (ESI) was performed in positive ion mode with nitrogen as the nebulizing, turbo spray, and curtain gas with the optimum values set at 50, 50, and 30 psi, respectively. The turbo gas temperature was set at 550 °C and the ESI needle voltage was adjusted to 5500 V. Multiple reaction monitoring (MRM) was employed using nitrogen as the collision gas with a dwell time of 100 ms for each transition. Data were acquired by Analyst 1.5.2 software.

2.5. Method validation

Validation of the assay was carried out in accordance with the currently accepted US Food and Drug Administration's bioanalytical method validation guidelines [16] with respect to the linearity, sensitivity, precision, accuracy, recovery, and stability. Specificity was evaluated from six different sources of blank human plasma to ensure that no interfering peaks were present at the respective

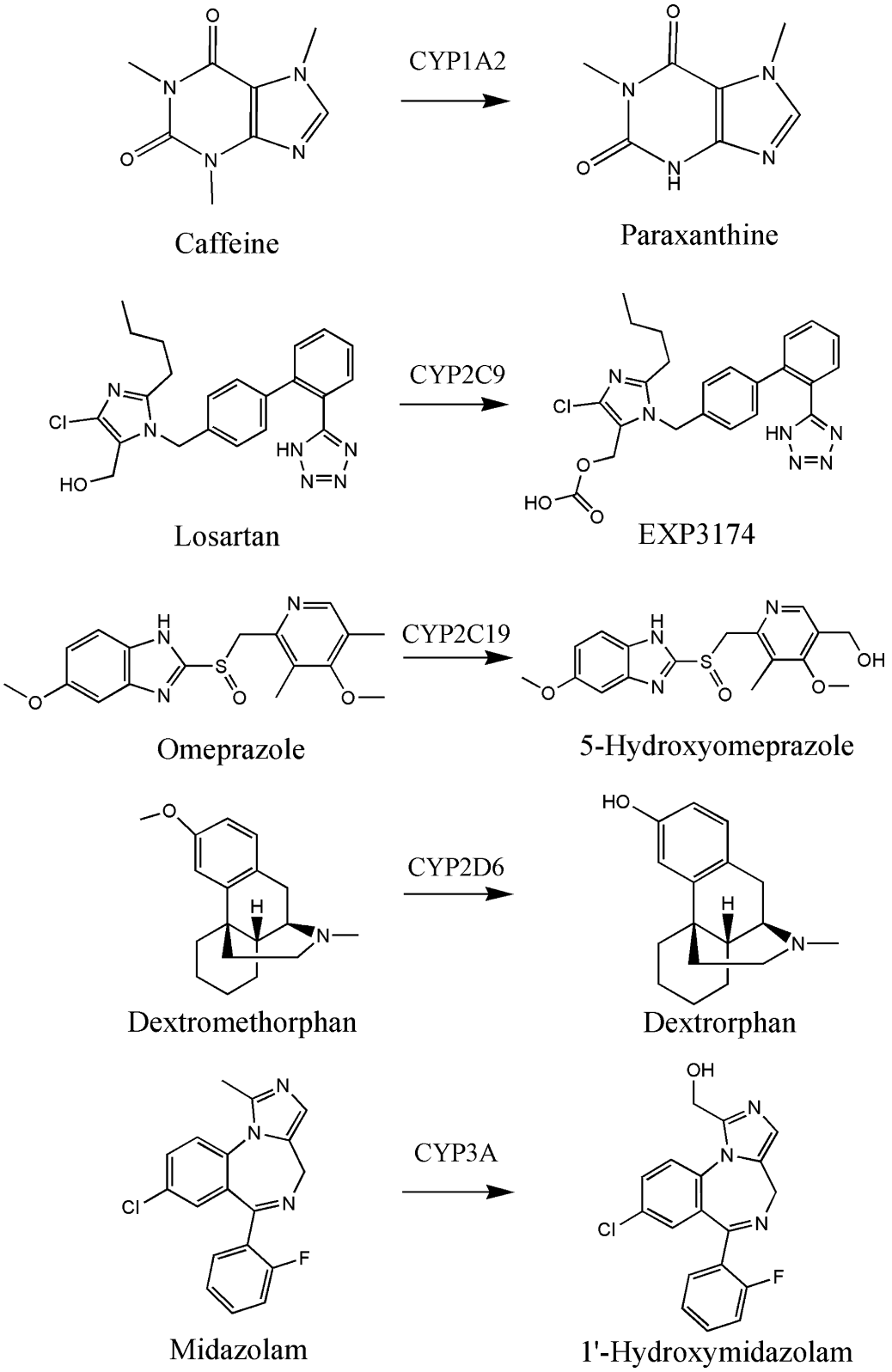

Fig. 1. Chemical structures of CYP isoform probe drugs used in this study.

peak regions of the analytes. Visible interferences were tested with blank plasma samples and plasma samples spiked with the analytes at the lower limit of quantitation (LLOQ). Calibration curves were constructed using six calibration standards for 3 different days and were fitted by a weighed least-squares linear regression. The regression parameters were calculated by a weighted least-squares linear-regression analysis for the analytes. To assess linearity, acceptable deviations of the mean calculated concentrations over three runs were set at ±15% of the nominal concentration except at the LLOQ, when a deviation of ±20% was permitted.

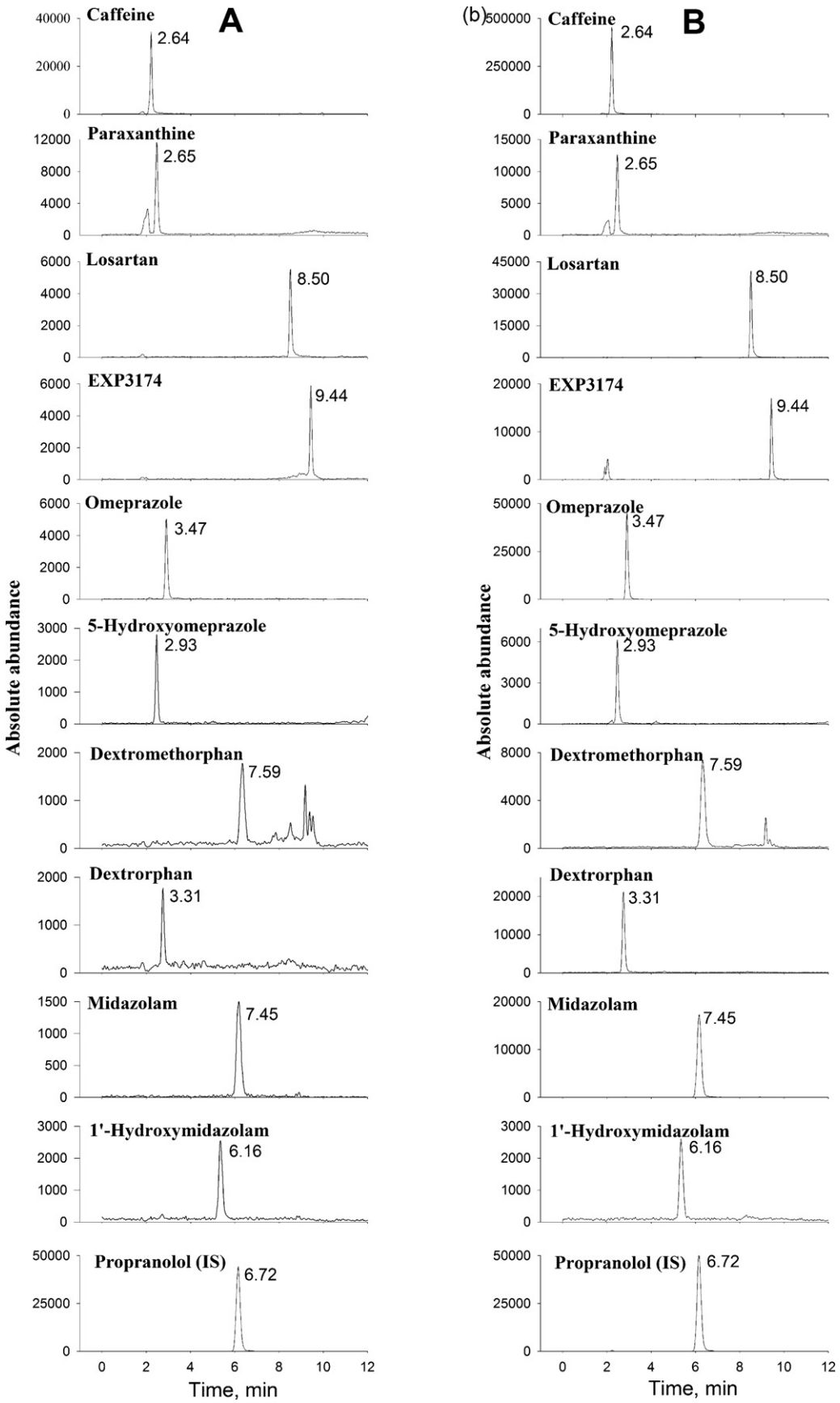

Fig. 2. SRM chromatograms of the probe drugs, their CYP isoform-selective metabolites and the internal standard in (A) plasma samples spiked with low QC concentrations and (B) plasma samples collected from a volunteer 2 h after dosing.

Table 1
MRM transitions and MS fragmentation parameters for the analytes and internal standard (IS).

Analyte	Polarity	Precursor (*m/z*)	Product (*m/z*)	DP (V)	CE (eV)	CXP (V)
Caffeine	+	195.0	138.3	180	30	10
Paraxanthine	+	181.0	124.4	180	30	10
Losartan	+	423.2	207.1	180	34	10
EXP3174	+	437.2	235.1	100	24	10
Omeprazole	+	346.2	198.1	100	17	8
5-Hydroxyomeprazole	+	362.2	214.1	100	17	4
Dextromethorphan	+	272.2	171.2	140	50	8
Dextrorphan	+	258.2	157.2	140	50	8
Midazolam	+	326.2	291.2	140	40	8
1′-Hydroxymidazolam	+	342.2	324.1	100	40	6
Propranolol	+	261.0	116.6	100	24	8

The accuracy and precision of the assay were assessed by analyzing QC samples under four different concentrations in six replicates on the same day and on 6 consecutive days for intra- and inter-day precision and accuracy, respectively. Precision was calculated as the relative standard deviation (RSD), and accuracy was assessed as the percent bias from the nominal concentration. Acceptable limits for intra- and inter-day precision and accuracy were set at ±15%, except at the LLOQ, when they were set at ±20%.

Recovery and matrix effects were assessed at three concentrations by comparing the peak areas of triplicate runs at each concentration for the analyte standards in methanol and the standard solutions spiked before and after solvent extraction in human plasma. The matrix effect was evaluated by comparing the mean peak area of an analyte spiked post-extraction to the mean peak area of an equivalent concentration of the same analyte standard in methanol. To further test whether a plasma matrix can cause any ion suppression, each drug solution (10 ng/mL) was infused post-column at a flow of 10 μL/min, resulting in constant production intensity. Then, plasma extracts were injected onto the column and changes in ion intensity were monitored to evaluate ion suppression as described in Ref. [17].

The stability of the analytes in plasma under different conditions was assessed at two QC levels ($n = 3$). The freeze–thaw stability was determined after three freeze (−80 °C) and thaw (20 °C) cycles and short-term stability was determined after 4 h at room temperature. To assess autosampler stability, extracts were placed in the autosampler at 5 °C for 24 h prior to analysis. An acceptable stability was defined as <15% loss of the initial drug concentration.

2.6. Application of the assay

The clinical applicability of the present method was evaluated by analyzing plasma taken from 13 volunteers who received oral doses of caffeine (10 mg), losartan (2 mg), omeprazole (200 μg), dextromethorphan (2 mg), and midazolam (100 μg). Volunteers were hospitalized 1 day before dosing and administered a single capsule containing the drug mixture at 09:00 the following day. Caffeine-containing diets were strictly restricted for 72 h prior to dosing. Blood samples were collected in tubes containing sodium heparin at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 h after dosing. The plasma samples were taken after centrifugation at 3000 rpm for 10 min and stored at −80 °C until analysis.

3. Results and discussion

3.1. LC–MS/MS method development

In a previous report, we described the "Inje cocktail," which consisted of caffeine, losartan, omeprazole, dextromethorphan, and midazolam as probes for CYP1A2, 2C9, 2C19, 2D6, and 3A4, respectively (Fig. 1) [9]. The usefulness of this 5-in-one approach for DDI studies was demonstrated and no significant interactions were observed among the drugs administered. However, that method required a dual analytical approach to quantitate dextromethorphan and dextrorphan. The 8-h urinary ratio of dextromethorphan/dextrorphan was used while the remaining analytes were measured in plasma. The goal of the current study was to develop and validate a reliable LC–MS/MS method to simultaneously detect all of the probe drugs and their metabolites in a single biological fluid with sufficient sensitivity to quantitate levels 10–100 times lower than those in therapeutic doses.

All the analytes in this study generated a prominent, protonated molecular ion $[M+H]^+$ in positive-ion mode; the most abundant fragment ions were chosen for MRM. The MRM transitions and optimized, collision-induced dissociation conditions are described in Table 1.

Sample preparation and chromatographic conditions were optimized for simple, rapid, and practical quantitative analysis. Because of the extremely high sensitivity of LC–MS/MS for these analytes, solvent extractions from relatively large plasma volumes (0.5 mL) were used in the assay. Omeprazole is unstable unless stored and handled at basic pH [18], which indicated a preference for basic media in the omeprazole analysis. Losartan and EXP3174 were more efficiently extracted under acidic conditions [19]. Therefore, a dual liquid–liquid extraction method was adopted to accommodate the different pH conditions. The plasma was initially extracted

Table 2
Calibration curves, linearity, and sensitivity of the assay.

Analyte	Retention time	Calibration range (ng/mL)	Correlation coefficient (R^2)	LLOQ (ng/mL)
Caffeine	2.2	5–500	0.9992 ± 0.0006	5
Paraxanthine	2.2	5–500	0.9994 ± 0.0004	5
Losartan	8.5	0.1–40	0.9972 ± 0.0013	0.1
EXP3174	9.4	0.1–40	0.9976 ± 0.0010	0.1
Omeprazole	2.9	0.05–20	0.9986 ± 0.0014	0.05
5-Hydroxyomeprazole	2.5	0.05–20	0.9990 ± 0.0004	0.05
Dextromethorphan	6.3	0.008–0.8	0.9997 ± 0.0003	0.008
Dextrorphan	2.7	0.008–0.8	0.9993 ± 0.0007	0.008
Midazolam	6.2	0.01–1	0.9997 ± 0.0002	0.01
1′-Hydroxymidazolam	5.3	0.04–4	0.9997 ± 0.0002	0.04

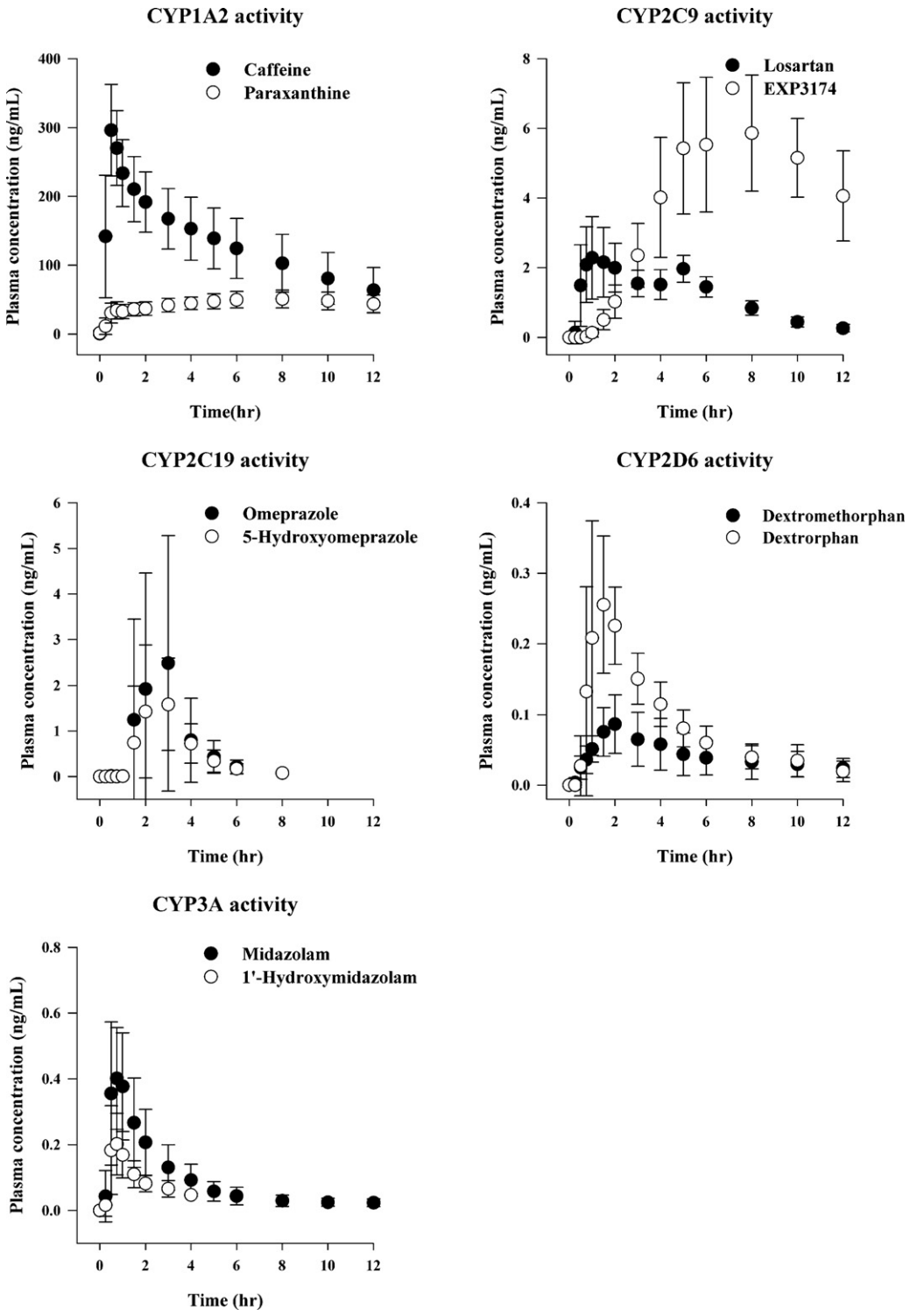

Fig. 3. Mean plasma concentration–time profiles of CYP substrates and their metabolites in volunteers after administration of cocktail drugs.

with ethylacetate at neutral pH, followed by a second extraction after acidification of the plasma with 0.1 N HCl. This resulted in high extraction recoveries for all of the drugs and metabolites (data not shown). In addition, light exposure was minimized during sample preparation and storage because midazolam is reported to be photodegraded by 10% within 1 h at pH 6.4 [20].

Several commercially available C_{18} columns were evaluated to optimize the chromatographic peak shape, peak width, and separation resolution. The Xbridge MS C_{18} column generated a narrow, relatively symmetric peak, with an optimal resolution under a gradient elution. When organic modifiers and acid modifiers were investigated, the combination of acetonitrile with 0.1% formic acid as a modifier resulted in higher ionization efficiency and better chromatographic separation than the combinations of acetonitrile and acetic acid, and methanol and formic acid. Under optimized chromatographic conditions, all of the target drugs were separated and analyzed within a 12-min run time (Table 2).

Table 3
Intra- and inter-day precision and accuracy.

Analyte	Nominal concentration (ng/mL)	Intraday (n = 6)			Inter-day (n = 6)		
		Measured (ng/mL)	RSD (%)	Accuracy (%)	Measured (ng/mL)	RSD (%)	Accuracy (%)
Caffeine	5.00	5.03 ± 0.57	11.3	100.6	4.37 ± 0.26	6.0	87.4
	15.0	13.8 ± 0.87	6.3	92.2	14.0 ± 0.74	5.3	93.2
	60.0	52.6 ± 1.23	2.3	87.7	56.3 ± 2.93	5.2	93.8
	300	274 ± 12.5	4.6	91.3	271 ± 12.6	4.6	90.2
Paraxanthine	5.00	4.84 ± 0.21	4.3	96.8	4.43 ± 0.24	5.5	88.6
	15.0	13.7 ± 1.01	7.4	91.5	13.6 ± 0.69	5.0	90.8
	60.0	51.9 ± 0.55	1.1	86.5	55.1 ± 2.22	4.0	91.9
	300	272 ± 11.3	4.1	90.8	263 ± 6.02	2.3	87.6
Losartan	0.10	0.11 ± 0.00	4.2	108.4	0.10 ± 0.01	7.1	99.9
	0.30	0.31 ± 0.02	6.7	102.1	0.30 ± 0.03	9.6	99.9
	2.40	2.31 ± 0.17	7.2	96.2	2.35 ± 0.15	6.3	97.8
	24.0	22.8 ± 0.72	3.1	95.0	22.4 ± 1.08	4.8	93.1
EXP3174	0.10	0.10 ± 0.01	12.5	102.5	0.11 ± 0.01	8.3	105.2
	0.30	0.29 ± 0.02	7.8	97.8	0.30 ± 0.03	9.5	99.3
	2.40	2.32 ± 0.14	5.9	96.8	2.38 ± 0.27	11.2	99.1
	24.0	24.5 ± 1.33	5.4	102.1	22.2 ± 1.50	6.7	92.5
Omeprazole	0.05	0.05 ± 0.00	7.4	105.3	0.05 ± 0.00	9.1	107.0
	0.15	0.15 ± 0.01	9.8	98.2	0.15 ± 0.01	8.8	102.3
	1.20	1.09 ± 0.06	5.3	90.5	1.18 ± 0.07	5.8	98.1
	12.0	11.1 ± 0.44	4.0	92.2	11.0 ± 0.59	5.4	91.5
5-Hydroxyomeprazole	0.05	0.05 ± 0.00	7.0	93.6	0.05 ± 0.00	8.4	99.6
	0.15	0.14 ± 0.01	8.1	94.8	0.14 ± 0.01	8.6	96.5
	1.20	1.05 ± 0.03	3.0	88.0	1.13 ± 0.07	6.2	93.7
	12.0	10.7 ± 0.29	2.8	89.4	10.8 ± 0.75	6.9	90.4
Dextromethorphan	0.01	0.01 ± 0.00	10.5	96.2	0.01 ± 0.00	11.2	94.2
	0.02	0.02 ± 0.00	4.0	91.1	0.02 ± 0.00	7.6	99.1
	0.10	0.09 ± 0.00	4.4	90.6	0.09 ± 0.01	6.1	93.0
	0.48	0.46 ± 0.03	7.2	95.0	0.45 ± 0.03	6.3	93.7
Dextrorphan	0.01	0.01 ± 0.00	7.8	96.0	0.01 ± 0.00	8.3	96.1
	0.02	0.02 ± 0.00	6.2	95.2	0.03 ± 0.00	4.9	104.7
	0.10	0.09 ± 0.00	2.0	91.4	0.09 ± 0.01	6.8	96.5
	0.48	0.44 ± 0.02	4.2	92.5	0.46 ± 0.03	6.9	94.9
Midazolam	0.01	0.01 ± 0.00	7.8	99.1	0.01 ± 0.00	13.8	96.0
	0.03	0.03 ± 0.00	7.5	96.5	0.03 ± 0.00	8.7	97.2
	0.12	0.11 ± 0.01	6.3	89.0	0.11 ± 0.01	8.3	94.9
	0.60	0.56 ± 0.02	4.0	94.0	0.57 ± 0.02	4.2	95.0
1′-Hydroxymidazolam	0.04	0.04 ± 0.00	3.6	94.7	0.03 ± 0.00	11.7	87.0
	0.12	0.12 ± 0.01	8.8	96.1	0.12 ± 0.01	6.7	97.8
	0.48	0.44 ± 0.03	6.3	91.2	0.46 ± 0.03	7.3	96.1
	2.40	2.30 ± 0.12	5.0	95.7	2.28 ± 0.09	3.9	95.1

In addition, separation of isobaric metabolites should be achieved in case of omeprazole and midazolam due to possible cross-talk. Under chromatographic conditions, 3-hydroxyomeprazole, 4-hydroxyomeprazole, and omeprazole sulfone were separated with retention times of 2.84, 1.98, and 3.89 min, respectively. 1′-Hydroxymidazolam and 4-hydroxymidazolam were also successfully separated with retention times of 4.23 and 3.80 min, respectively. Typical chromatograms of the probe drugs and their CYP isoform selective metabolites are presented in Fig. 2.

3.2. Method validation

Specificity and selectivity were investigated by preparing and analyzing blank samples from six different batches of human plasma. No interference was observed in drug-free plasma samples at the retention times of the target drugs. Additionally, no carryover effects were observed in our system. Typical chromatograms for plasma spiked with low QC levels of all analytes and plasma collected from one patient 2 h after dosing are shown in Fig. 2. Calibration curves for each drug and metabolite were linear at 5–500 ng/mL for caffeine and paraxanthine, 0.1–40 ng/mL for losartan and EXP3174, 0.05–20 ng/mL for omeprazole and 5-hydroxyomeprazole, 0.008–0.8 ng/mL for dextromethorphan and dextrorphan, 0.01–1 ng/mL for midazolam, and 0.04–4 ng/mL for 1′-hydroxymidazolam with correlation coefficients (R^2) greater than 0.997. The best-fit line of the calibration curve for each analyte was obtained by using a weighting factor of $1/x$ for caffeine, paraxanthine, dextromethorphan, dextrorphan, midazolam, and 1′-hydroxymidazolam. A weighting factor of $1/x^2$ was applied to calibration curves for losartan, EXP3174, omeprazole, and 5-hydroxyomeprazole due to their wide calibration range.

For all of the drugs and metabolites tested, intraday CVs were less than 12.5% and intraday accuracies were between 86.5% and 108.4% at the LLOQ, low, middle, and high concentrations. The inter-day data were also accurate and reproducible with CVs less than 13.8% and accuracies between 87.0% and 107.0%. The detection limits for drugs and metabolites ranged from 0.008 to 5.0 ng/mL with CVs and accuracies less than 20% (Table 3).

The overall extraction recovery was assessed by comparing the peak areas of extracted plasma samples at low, middle, and high QC levels to the peak areas of neat solutions spiked at the corresponding concentrations. The extraction recovery from human plasma was higher than 63% for all analytes. These values were from

Table 4
Stability of analytes and the IS.

Analyte	Nominal concentration (ng/mL)	4-h short-term stability	24-h post-preparative stability (5 °C)	Freeze–thaw stability (−80 °C/room temperature)
Caffeine	15	90.8	100.6	107.4
	300	107.3	100.6	103.8
Paraxanthine	15	88.4	100.6	105.5
	300	107.4	100.8	104.1
Losartan	0.3	85.2	109.7	102.2
	24	98.6	106.1	107.7
EXP3174	0.3	111.9	115.1	113.8
	24	104.1	114.4	107.7
Omeprazole	0.15	95.9	112.3	99.0
	12	103.4	101.2	97.7
5-Hydroxyomeprazole	0.15	89.5	104.7	108.3
	12	104.8	104.9	106.1
Dextromethorphan	0.024	100.0	102.4	94.4
	0.48	101.1	102.6	102.7
Dextrorphan	0.024	94.2	102.5	101.6
	0.48	106.5	104.1	99.1
Midazolam	0.03	100.1	104.3	96.8
	0.6	103.7	103.7	104.9
1′-Hydroxymidazolam	0.03	101.0	112.0	105.2
	0.6	101.7	111.7	107.5

The experiment was performed in triplicate.

combined contributions from recovery loss and matrix suppression. The extraction recovery was also consistent for each analyte over the concentration ranges, suggesting that extraction efficiency was independent of analyte concentration. To further determine whether matrix interference affected the analyses, blank plasma sample was injected into the LC–MS/MS system while the individual analyte was introduced continuously by post-column infusion. No significant matrix interference was observed near the retention times of all analytes tested (data not shown).

Stock solutions of the drugs and metabolites in methanol were stable for at least 3 weeks at 4 °C and for 4 months at −80 °C. No significant degradation, defined as any deviation outside of ±15% of the nominal concentration, of any of the analytes occurred in human plasma after short-term storage for 6 h at room temperature, two freeze–thaw cycles, or posttreatment storage for 24 h at 5 °C (Table 4).

3.3. Clinical application

The developed method was used to determine concentrations of the target drugs and their CYP isoform-specific metabolites in human plasma after concomitant oral administration of five drugs to 13 healthy volunteers. The doses given to the volunteers were 10 times (caffeine) to 100 times (omeprazole) lower than their therapeutic doses. Fig. 3 shows the mean plasma concentration–time curves for five CYP isoform probe drugs and their representative metabolites. Caffeine/paraxanthine, losartan/EXP3174, and dextromethorphan/dextrorphan were detected over the 12-h time period. Omeprazole and 5-hydroxyomeprazole were detected 1.5 h after dosing due to enteric coating with maximum concentration (C_{max}) values of 2.4 and 1.9 ng/mL, respectively. 1′-Hydroxymidazolam was detected within 6 h with C_{max} and area under concentration (AUC_t) values of 0.2 ng/mL and 0.2 ng h/mL, respectively. Thus, the analytical method described herein was sufficiently sensitive to use in low-dose pharmacokinetic studies.

4. Conclusions

A highly sensitive and reliable LC–MS/MS method was developed for simultaneous quantitation of CYP isoform probe drugs and their metabolites in human plasma. To the best of our knowledge, this method is most sensitive (at least 20 times) and simple (dual solvent extraction) compared to previously report simultaneous quantitation methods for cocktail drug interaction studies. The present method affords the sensitivity, accuracy and precision necessary for pharmacokinetic evaluation of five drugs and their corresponding CYP isoform-specific metabolites in humans who received 10–100 times lower doses than conventional therapeutic doses. The present method was proven to be suitable for minidose cocktail trials for DDIs.

Acknowledgments

This work was supported by the Basic Research Program through the National Research Foundation of Korea (2010-0005370), Ministry of Education, Science and Technology, Korea. K.S. Oh is technical research personnel appointed by Korea Ministry of National Defense.

References

[1] D.W. Nebert, D.W. Russell, Lancet 360 (2002) 1155.
[2] S. Zhang, N. Song, Q. Li, H. Fan, C. Liu, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 871 (2008) 78.
[3] H.G. Xie, R.B. Kim, Annu. Rev. Pharmacol. Toxicol. 41 (2001) 815.
[4] A.N. Nafziger, J.S. Bertino Jr., Clin. Pharmacol. Ther. 82 (2007) 379, author reply 380.
[5] E. Tanaka, N. Kurata, H. Yasuhara, J. Clin. Pharm. Ther. 28 (2003) 157.
[6] R.F. Frye, G.R. Matzke, A. Adedoyin, J.A. Porter, R.A. Branch, Clin. Pharmacol. Ther. 62 (1997) 365.
[7] S. Chainuvati, A.N. Nafziger, J.S. Leeder, A. Gaedigk, G.L. Kearns, E. Sellers, Y. Zhang, A.D. Kashuba, E. Rowland, J.S. Bertino Jr., Clin. Pharmacol. Ther. 74 (2003) 437.
[8] M. Christensen, K. Andersson, P. Dalen, R.A. Mirghani, G.J. Muirhead, A. Nordmark, G. Tybring, A. Wahlberg, U. Yasar, L. Bertilsson, Clin. Pharmacol. Ther. 73 (2003) 517.

[9] J.Y. Ryu, I.S. Song, Y.E. Sunwoo, J.H. Shon, K.H. Liu, I.J. Cha, J.G. Shin, Clin. Pharmacol. Ther. 82 (2007) 531.
[10] U. Fuhr, A. Jetter, J. Kirchheiner, Clin. Pharmacol. Ther. 81 (2007) 270.
[11] Y. Sugiyama, S. Yamashita, Adv. Drug Deliv. Rev. 63 (2011) 494.
[12] S.K. Balani, N.V. Nagaraja, M.G. Qian, A.O. Costa, J.S. Daniels, H. Yang, P.R. Shimoga, J.T. Wu, L.S. Gan, F.W. Lee, G.T. Miwa, Drug Metab. Dispos. 34 (2006) 384.
[13] K.A. Woelkart, M. Kollroser, L. Li, Anal. Bioanal. Chem. 400 (2011) 2371.
[14] O.Q. Yin, S.S. Lam, C.M. Lo, M.S. Chow, Rapid Commun. Mass Spectrom. 18 (2004) 2921.
[15] S. Ghassabian, M. Chetty, B.N. Tattam, Ther. Drug Monit. 31 (2009) 239.
[16] FDA Guidance for Industry, Bioanalytical Method Validation, May 2001, Available from: www.fda.gov.
[17] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[18] V.A. Frerichs, C. Zaranek, C.E. Haas, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 824 (2005) 71.
[19] A. Soldner, H. Spahn-Langguth, E. Mutschler, J. Pharm. Biomed. Anal. 16 (1998) 863.
[20] R. Andersin, M. Mesilaakso, J. Pharm. Biomed. Anal. 13 (1995) 667.