Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 856 (2007) 318–327
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Multi-component plasma quantitation of anti-hyperglycemic pharmaceutical compounds using liquid chromatography–tandem mass spectrometry

Ming Wang *, Irina R. Miksa *

Merck & Co., Inc. Safety Assessment, Biochemical Toxicology, West Point, PA 19468, United States

Received 8 February 2007; accepted 22 June 2007
Available online 6 July 2007

Abstract

Type-2 diabetes is a disorder characterized by disrupted insulin production leading to high blood glucose levels. To control this disease, combination therapy is often used. Hypoglycemic agents such as metformin, glipizide, glyburide, repaglinide, rosiglitazone, nateglinide, and pioglitazone are widely prescribed to control blood sugar levels. These drugs provide the basis for the development of a quantitative multianalyte bioanalytical method. As an example, a highly sensitive and selective multi-drug method based on liquid chromatography tandem mass spectrometry (LC–MS/MS) was developed. This rapid, automated method consists of protein precipitation of 20 μL of plasma coupled with gradient HPLC elution of compounds using 10 mM ammonium formate buffer and 0.1% formic acid in acetonitrile as the mobile phases. MS/MS detection was performed using turbo ion spray in the positive ion multiple reaction monitoring (SRM) mode. A lower limit of quantitation (LLQ) in a range of 1.0–5.0 ng/mL was achieved for all analytes. The linearity of the method was observed over a 500-fold dynamic range. Drug recoveries ranged from 86.2 to 94.2% for all analytes of interest. Selectivity, sample dilution, intra-day and inter-day accuracy and precision, and stability assessment were evaluated for all compounds.

Keywords: Chromatography; Mass spectrometry; Hypoglycemic; Drugs; GLP; Analytical; Chemistry

1. Introduction

Type-2 diabetes is a long-term metabolic disorder wherein the body becomes resistant to the effects of insulin, a hormone that regulates sugar absorption [1]. Treatment of type-2 diabetes (non-insulin dependent) is now possible with orally administered hypoglycemic agents that help reduce blood sugar levels. Five major classes of chemically diverse hypoglycemic drugs with different mechanisms of action have been developed for administration to patients. These are known as sulfonylureas (glipizide, glyburide, glipermide, chlorpropamide and tolazamide), biguanides (metformin), thiazolidinediones (pioglitaone and rosiglitazone), meglitinides (repaglinide and nateglinide), and alpha-glucosidase inhibitors (acarbose and miglitol) [2].

Oral hypoglycemic drugs prescribed as monotherapy have not provided enough hypoglycemic control for type-2 diabetic patients. For this reason, combination therapy is becoming a more prevalent method for achieving satisfactory blood glucose levels [3–7]. The majority of combination medicines available to date are based on metformin co-administration. Examples of combination medication include metformin with sulfonylureas such as glyburide (*GlucoVance*) and glipizide (*Metaglip*), or thiazolidinediones resulting in *ActoplusMet* (with pioglitozone) and *Avandamet* (with rosiglitazone) [8–12]. Drug monitoring during combination therapy is an important process for titrating the appropriate dosing control and diagnostic purposes [13–15]. Since combination therapies are becoming widespread in application, it is of importance to develop methods applicable to the pre-clinical development of potential combinations of these classes of drugs. Therefore, sensitive, rapid and reliable methodology is required for simultaneous multianalyte quantitation of oral hypoglycemic drugs in the plasma of animals used for preclinical development.

Available reports suggest that liquid chromatography–mass spectrometry (LC–MS) and LC with UV detection in conjunction with solid phase extraction (SPE) or liquid–liquid extraction

* Corresponding authors. Tel.: +1 215 652 7599; fax: +1 215 993 7537.
E-mail address: irina_miksa@merck.com (I.R. Miksa).

1570-0232/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2007.06.037

(LLE) are the most commonly used methods for detection and quantitation of anti-diabetic drugs in biological samples [16–22].

Based upon the need for co-administration of hypoglycemic agents, several multi-drug screening procedures have been developed. The reported methods are applicable to analysis of human plasma, equine plasma and urine, and tablet formulations [23–28]. Methods include LC procedures for metformin and glipizide with a lower limit of quantitation (LLQ) of 5.0 and 22.5 ng/mL [23], or metformin and glimepride with a LLQ of 22 and 39 ng/mL, respectively [24]. Methods based on LC/MS/MS have been reported for metformin and gliclazide (LLQ range of 7.8–10.0 ng/mL) [25]. Simultaneous quantitation of eight sulfonylureas based on LC–MS/MS was also reported utilizing LLE and obtaining LLQ in the range of 7.8–78 ng/mL [26]. Determination of glipizide and/or rosiglitazone, tolazamide and repaglinide using LC/MS/MS was reported for equilibrium dialysis and LLE with an LLQ range of 1.0–10.0 ng/mL [27–28]. Anti-diabetic drugs have also been included in the context of forensic and clinical toxicology drug screening [29–30].

This article describes a simple, rapid, automated, reproducible and reliable multi-residue quantitation method for seven oral hypoglycemic drugs in monkey plasma. Compounds under investigation belong to four different classes and possess different chemical structures (Fig. 1). Sample preparation omits cumbersome SPE or LLE methodologies, is automated, and drug

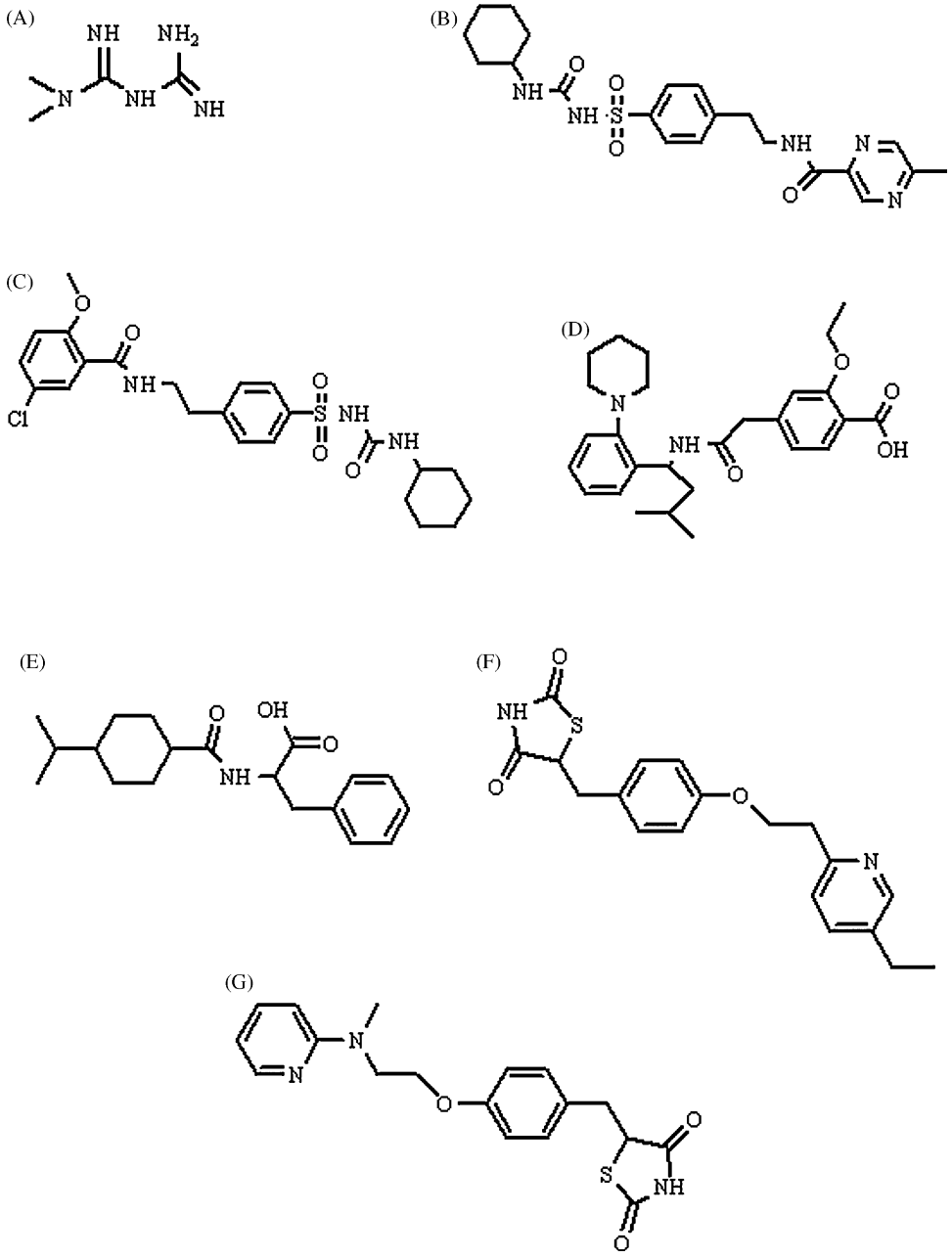

Fig. 1. Chemical structures of seven oral hypoglycemic drugs. (A) Metformin, (B) glipizide, (C) glyburide, (D) repaglinide, (E) nateglinide, (F) pioglitazone, and (G) rosiglitazone.

elution is achieved without the need for derivatization. Product ion mass spectral fragmentation patterns are obtained in the positive ion mode of electrospray ionization (ESI) and reported for each compound. Accurate quantitation of all analytes is achieved over a wide range of concentrations. All assessments were based on a 20 μL sample volume and evaluated based upon most of the criteria stated in the U.S. Food and Drug Administration Center for Drug Evaluation and Research (FDA/CDER) guidelines for Bioanalytical Method Validation (May, 2001).

2. Experimental

2.1. Material and reagents

Glipizide (GLI), glyburide (GLY) and repaglinide (REP) were purchased from Sigma–Aldrich, Inc. Rosiglitazone (ROS) was purchased from Alexis Biochemicals. Pioglitazone (PIO) was obtained from ChemPacific Co. Repaglinide-ethyl-d5 (IS-1), nateglinide-phenyl-d5 (IS-2) and nateglinide (NAT) were obtained from Toronto Research Chemicals, Inc. Metformin (MET) and IS-3, internal standard compound used in Zeng et al. [31], were prepared at Merck & Co., Inc.

Tecan robotics supplies such as pipette tips, reagent troughs, 96-deep-well polypropylene plates were purchased from Molecular Bioproducts and Porvair, Inc. 96-deep-well (2 mL) Strata™ Impact protein precipitation plates was obtained from Phenomenex, Inc.

Acetonitrile (ACN; Optima grade), methanol (MeOH), isopropanol (IPA), 0.1% formic acid (FA) in ACN (HPLC grade), 96% FA and ammonium formate were purchased from Fisher Scientific. Ultra Pure water system from Millipore (Milli-Q RG and Milli-RO10) was used to generate water (H_2O) with resistivity of 18 MΩ cm.

2.2. Solutions

Standard diluent solution was prepared with 50% ACN and 50% H_2O (50:50 ACN: H2O). Needle wash and valve wash solutions used were standard diluent and 4:3:3 ratio of ACN:IPA:H_2O with 0.1% FA. Solution of 10 mM ammonium formate was prepared by adding 1.26 g of ammonium formate to 2 L of H_2O. All solutions were stored at room temperature.

2.3. LC–MS/MS conditions

A Sciex API 4000 triple quadrupole MS system was equipped with a Turbo Ionspray interface, binary pump and CTC-PAL autosampler with an external valve wash system. Mass spectral setting set to operate in positive-ion mode (ESI+) were: voltage at 4500 V, temperature at 550 °C, collision gas (N_2) was set at 4, curtain gas set at 40, ion source gases set at 60 and 50. The Thermo Fluophase PFP-reverse phase column (50 mm × 2.1 mm; 5 μm particle size) was equilibrated with the mobile phase (40% of 0.1%FA in ACN and 60% of 10 mM ammonium formate solution) at 0.6 mL/min prior to analysis. Injection sample volume was set to 10 μL with 5 μL loop volume. The LC profiles were developed at ambient temperature and the following gradient elution: 0–1.0 min, 40% of 0.1%FA in ACN; 1.0–3.5 min, to 95% of 0.1% FA in ACN; 3.51–5.50 min, back to 40% of 0.1% FA in ACN. IS-1 and IS-2 were used for determination of REP and NAT, respectively. IS-3 [31] was used for simultaneous detection and quantitation of all other drugs of interest. Retention times for all compounds and their corresponding MS transitions developed in the selective reaction monitoring (SRM) mode are summarized in Table 1.

2.4. Preparation of standard and quality control (QC) solutions

Individual stock standards for all compounds were prepared by weight at 1.0 mg/mL concentrations and at 1000 ng/mL in MeOH. Mixed working standard solutions at eight levels were prepared in standard diluent. Standards and QC samples were made from separate stock solutions. Standards were prepared over a range of 2.0–1000 ng/mL for MET, REP, GLY, PIO and ROS, 4.0–2000 ng/mL for GLI, and 10.0–5000 ng/mL for NAT. Three levels of mixed working QC solutions were prepared for all analytes. A mixed working IS solution was prepared containing IS-1 and IS-2 at 100 ng/mL and IS-3 at 1000 ng/mL in standard diluent. All standards were stored at 4 °C when not in use.

Table 1
Chromatographic and SRM transitions data for four classes of hypoglycemic drugs and three IS compounds

Full name	Abb.	RT (min)	Transition	
Analyte	Analyte		Q1 (precursor); *m/z*	Q3 (product ion); *m/z*
Metformin	MET	0.5	130	71
Glipizide	GLI	0.8	446	321
Glyburide	GLY	2.5	494	369
Repaglinide	REP	3.7	453	230
Repaglinide-ethyl-d5	IS-1	3.7	458	230
Nateglinide	NAT	1.8	318	166
Nateglinide-phenyl-d5	IS-2	1.8	323	125
Pioglitazone	PIO	2.1	357	134
Rosiglitazone	ROS	1.7	358	135
N/A	IS-3	1.5	390	156

Abb., abbreviation; RT, retention time; N/A, not applicable.

2.5. Sample extraction and analysis

Prior to fortification and extraction, a pool of control blank monkey plasma was vortexed and centrifuged at ambient temperature. A Tecan Genesis Freedom 200 equipped with a robotic manipulator arm, TeVacS vacuum system, Eppendorf thermomixer, and an 8-tip liquid handling arm equipped with disposable tip adapters was used in an automated protein precipitation sample extraction procedure. A Strata™ Impact protein precipitation plate coupled with a 96-deep-well (collection) plate was placed inside a vacuum manifold on the Tecan. Initially, 200 μL of acetonitrile was added to the protein precipitation plate. This was followed by the addition of the appropriate volume of standard diluent, 20 μL of monkey plasma and the appropriate amount of the IS solution. Preparation of standard and QC samples was based on 200 μL of ACN, 10 μL of mixed IS working solution, 20 μL of blank monkey plasma and 10 μL of appropriate mixed standard or QC working solution. The protein precipitation plate was then vortexed for 65 s and vacuum was applied to facilitate filtration. The filtrate was collected into the 96-deep-well collection plate and 10 μL of filtrate was injected to the LC–MS/MS system.

2.6. Accuracy and precision assessments

The intra-day accuracy and precision assessment was based on five individual calibration curves and five sets of QC samples. A valid QC set was defined as duplicate samples at concentrations at the low end (≤3 times LLQ), approximate to arithmetic middle and the upper end (≥75% of upper limit of quantitation, ULQ) of the intended calibration curve established for each analyte. An acceptable intra-day assessment for standard curves was reached when the mean calculated value at each standard concentration was 100 ± 15% the theoretical value while the precision (expressed as coefficient of variation, %CV) was not to exceed 15% (20% at LLQ). An acceptable intra-day assessment for QC samples was reached when the mean calculated value at each QC concentration was 100 ± 15% of the theoretical value with precision not exceeding 15%.

Inter-day accuracy and %CV assessments were based on a 3-day validation and established for QCs and LLQ. A specific and predefined QC set and LLQ from each day were used to assess inter-day accuracy (determined by comparing the found concentration vs. the nominal value) and %CV. An acceptable inter-day assessment was reached when the mean calculated accuracy of the QCs was 100 ± 15% and precision did not exceed 15%. Criteria for acceptable inter-day mean accuracy at the LLQ was 100 ± 20% with precision not exceeding 20%.

2.7. Sample matrix effects

Matrix effect was defined as the suppression or enhancement of ionization of analytes due to the presence of co-eluting matrix ions during LC/MS/MS analysis. A matrix factor (MF) was defined as the quantitative measure of matrix effects for each analyte in monkey plasma. MF was defined for each analyte of interest (and IS) as the peak area ratio between the analyte response when present in the matrix versus the analyte response in solvent (diluent) following the sample extraction procedure (see MF calculation).That is:

$$\text{MF(analyte)} = \frac{\text{(Peak Area of Analyte in Presence of Matrix)}}{\text{(Peak Area of Analyte in Solvent)}}$$

MF for each compound was normalized with corresponding IS response with the following expression:

$$\text{MF(IS normalized)} = \frac{\text{MF of Analyte}}{\text{MF of IS}}$$

Ion-suppression was considered to be taking place with an obtainable MF of less then 1.0 and ion-enhancement was considered for MF higher then 1.0. MF was considered acceptable as long as the IS normalized value was 0.8–1.2 or expressed as an accuracy of 100 ± 20%.

2.8. Extraction efficiency (recovery) and sample dilution

Extraction efficiency was expressed in terms of recovered concentration of analyte and IS added to a biological matrix prior to extraction (recovery QC) vs. concentration obtained with a biological sample where analyte and IS were added following extraction (reference QC). All analyses were performed in triplicate for each analyte at three concentrations based on mixed working solutions. Percent drug recovery with corresponding %CV was determined for each plasma sample fortified with all analytes of interest. All samples were prepared with Tecan robotics. Reference QC samples were extracted based on a 20 μL blank monkey plasma procedure as described above. After filtration of the extracted blank plasma sample, 10 μL of an appropriate mixed QC solution and 10 μL IS mixed working solutions were added. The plate was vortexed, sealed and sample analyzed by LC–MS/MS.

2.9. Sample dilution

The process of assaying a partial volume of a sample prepared at a concentration above the ULQ (sample dilution) was assessed with the specified method utilizing Tecan robotics. Sample dilution for a 20-fold double dilution assessment was performed in triplicate for blank monkey plasma fortified with all analytes at a concentration above ULQ and diluted twice with additional blank monkey plasma. Acceptable criteria were set for the accuracy of the mean concentration to 100 ± 15% of the nominal value with the %CV not to exceed 15%.

2.10. Stability

Stability assessments (defined as the resistance to change in concentration of an analyte in plasma under specific conditions) such as short-term temperature stability (STS), freeze–thaw stability (FTS) and final matrix (FM) stability were performed for all analytes.

STS and FTS determinations were based on analyte response of a biological matrix stability sample (BSS) prior to freezer storage versus analyte response for a BSS sample stored at −70 °C.

BSS samples were prepared at two levels of concentration, low (≤5 times LLQ) and high (≥70% of the ULQ). An acceptable BSS sample had a %CV not exceeding 15% on the day of sample preparation (defined as Day 0). The mean of the Day 0 calculated values based on measured concentrations of the BSS's for each analyte was used as the reference sample concentration for subsequent stability assessments. Triplicate BSS samples at each concentration were removed for analysis 24 h following specified storage. STS was assessed at each concentration for each analyte following a 5 h thaw at room temperature. The FTS was assessed by performing three freeze–thaw cycles for each low and high sample concentration. Stability of all samples was considered acceptable if the accuracy of the mean of each analyte at each concentration was within ±25% as compared to the reference sample concentration of the Day 0 BSS samples.

The FM sample was defined as the final extract of a biological sample fortified with all analytes of interest and IS that has undergone all sample preparation procedures. FM stability of all analytes and IS was determined in triplicate at two levels of concentration as compared to QC samples, lower end (≤3 times LLQ) and the upper end (≥75% of ULQ) of the intended calibration curve. FM samples were prepared by Tecan robotics as described in the QC sample preparation procedure

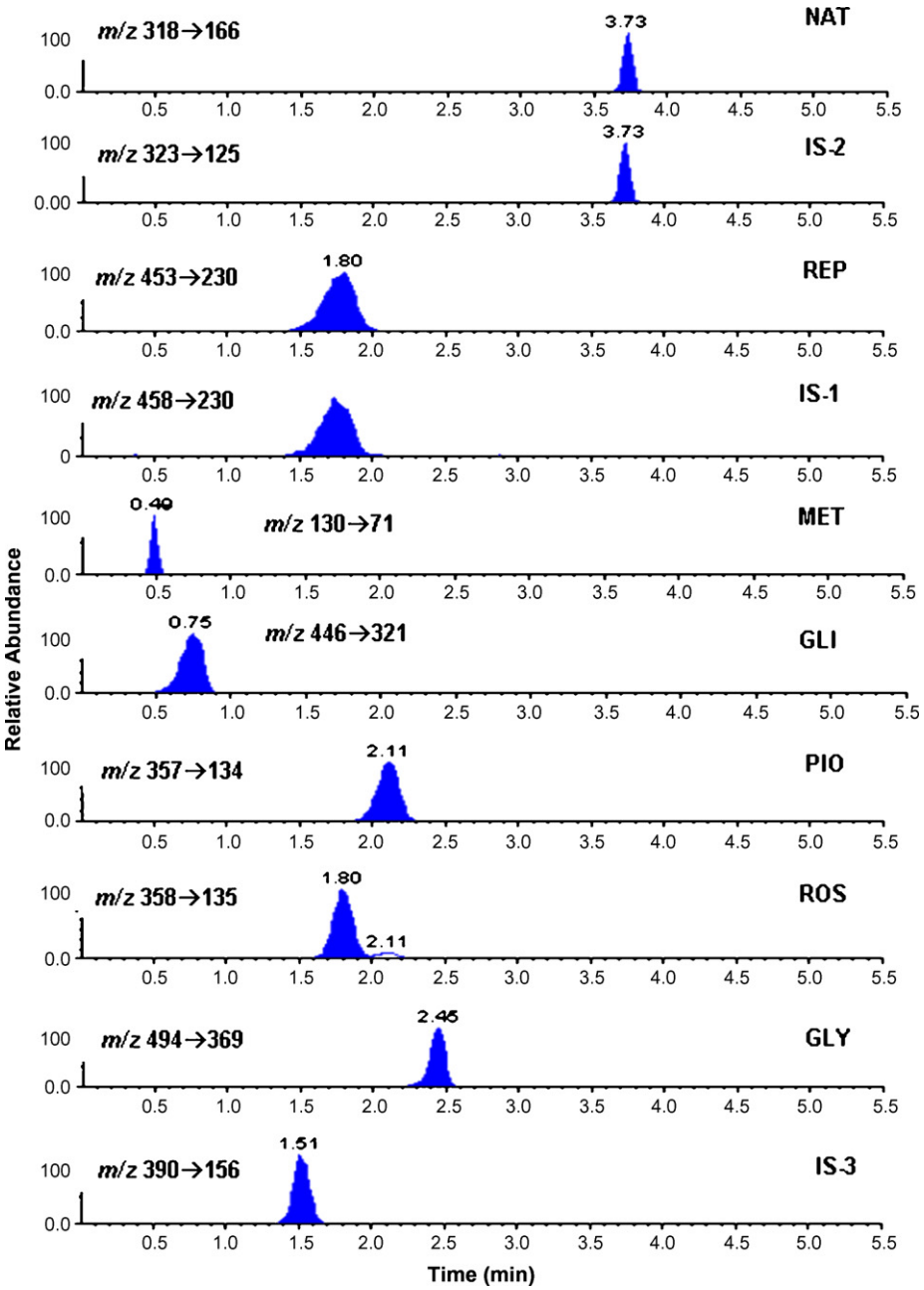

Fig. 2. Extracted-ion chromatograms for seven analytes and three IS compounds extracted from fortified monkey plasma. The concentration of each analyte was as follows: 100 ng/mL for IS-1 and IS-2, 1000 ng/mL for IS-3, 800 ng/mL GLI, 2000 ng/mL for NAT, 400 ng/mL for all other compounds. Transitions are shown with corresponding chromatograms.

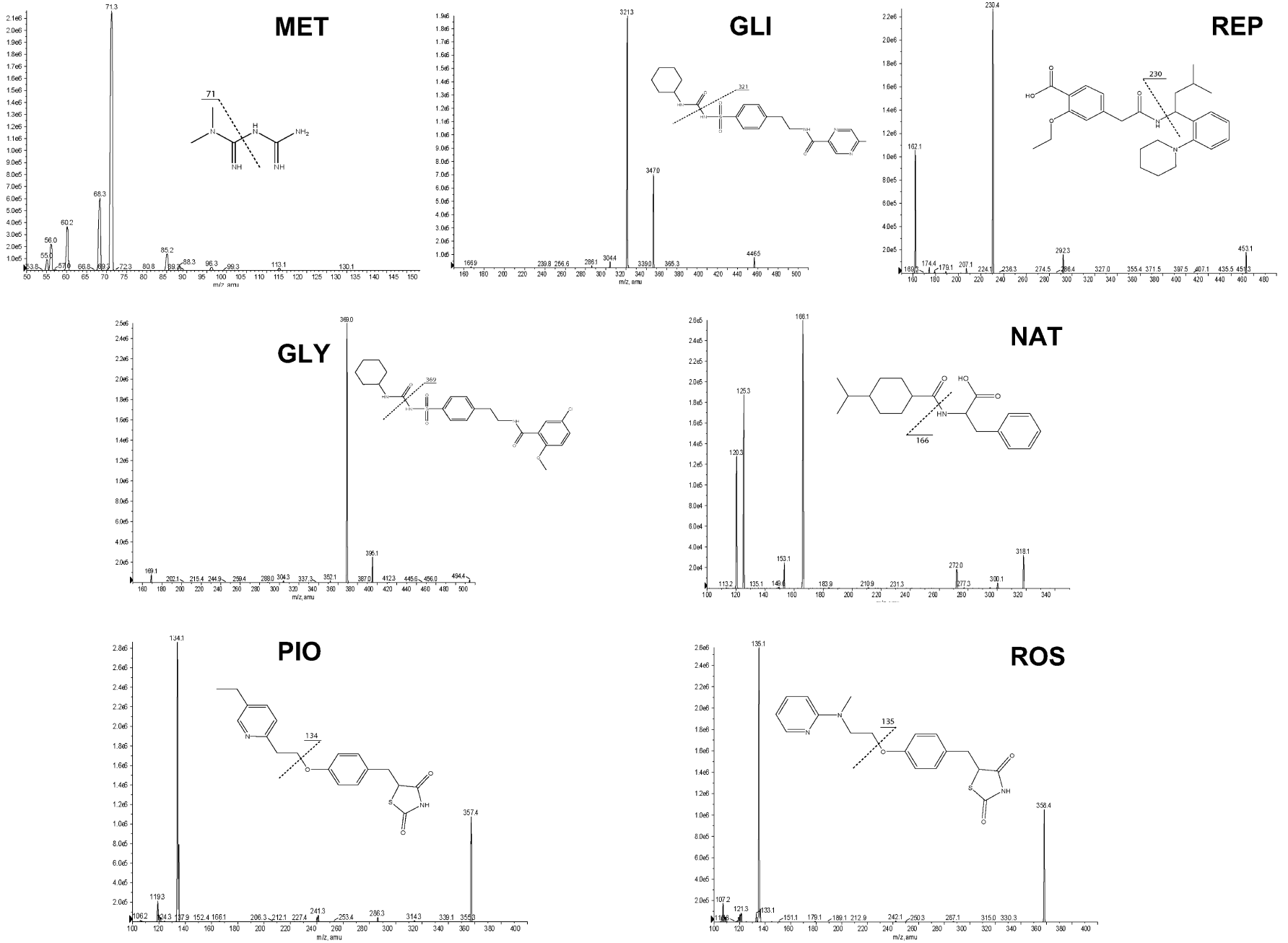

Fig. 3. Positive ESI product-ion spectra obtained for all compounds when present in individual stock standard solutions at 1000 ng/mL. Structures and proposed fragmentation to yield high abundance fragments are shown for each compound.

Table 2
Intra-day assay accuracy and precision for LLQ and ULQ ($n = 5$)

Analyte name	LLQ			ULQ		
	Concentration (ng/mL)	% Accuracy	% CV	Concentration (ng/mL)	% Accuracy	% CV
MET	1.00	98.7	10.7	500	92.1	2.96
GLI	2.00	99.0	2.97	1000	102	2.60
GLY	1.00	100	14.5	500	102	2.72
REP	1.00	98.9	6.55	500	101	1.90
NAT	5.00	100	9.92	2500	103	2.72
PIO	1.00	101	5.76	500	100	3.46
ROS	1.00	99.4	3.31	500	98.7	2.27

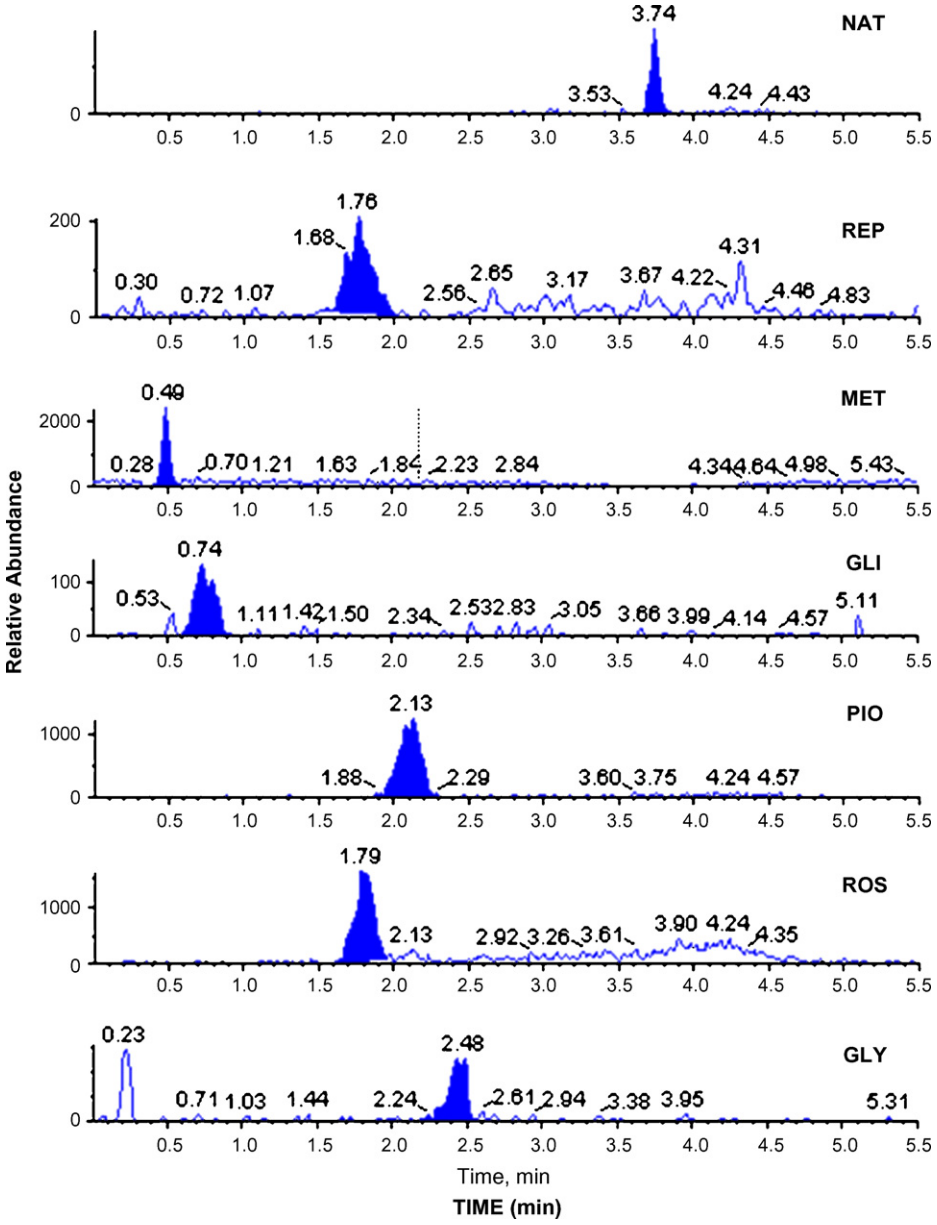

Fig. 4. Extracted-ion chromatogram for a monkey plasma LLQ sample based on a 20 μL plasma volume.

and allowed to remain in the 96-deep-well collection plate in the LC autosampler for at least 24 h. FM samples were injected onto the instrument and analyzed with a freshly prepared set of analytical reference standards (standard curve and two sets of QCs). The FM assessment was considered acceptable when the accuracy of the mean calculated concentration at each level for each analyte was ±15%. The FM stability interval was defined as the time between when the samples were placed onto the LC autosampler to the time when the last FM sample was injected for analysis.

3. Results and discussion

3.1. LC–MS/MS analysis

Seven different structurally diverse oral drugs for the treatment of Type-2 diabetes and three IS compounds were successfully separated from the solvent front and chromatographed within a 5.5 min LC run. Retention times for all analytes are summarized in Table 1. Extracted-ion SRM chromatograms for working standards of all analytes in monkey plasma (concentration of 800 ng/mL for GLI, 2000 ng/mL for NAT, and 400 ng/mL for all other analytes) are shown in Fig. 2. Signal intensity for all analytes in monkey plasma was found to be optimal in a positive mode of ESI. The Q1 mass spectrum of each analyte showed protonated molecular ions $[M+H]^+$ at m/z values summarized in Table 1. The product-ion spectrum for each analyte (Fig. 3) when present in its individual stock solution at 1000 ng/mL showed high abundance fragments at m/z of 71, 321, 369, 230, 166, 134 and 135 for MET, GLI, GLY, REP, NAT, PIO, and ROS, respectively. The SRM transitions (Q1 → Q3) for all analytes and IS compounds chosen for monitoring are listed in Table 1 and are in agreement with previously published work on GLI, ROS, MET, PIO, NAT and REP [25–28]. As seen from the selected m/z transitions of PIO and ROS, 357 → 134 and 358 → 135, respectively, chromatographic separation of these two compounds was favored in order to avoid any possible cross-channel interference. Baseline separation as well as appropriate peak shape and sensitivity was achieved using a Thermo Fluophase PFP reverse phase column and the 5.5 min gradient method (see Section 2 for details).

3.2. Method selectivity and matrix effects

Method selectivity towards endogenous monkey plasma matrix was tested in three different lots of blank plasma and no interferences were observed at the SRM for all analytes tested. Blank matrix sample and IS blank monkey plasma sample were prepared and included as a part of each calibration curve. In addition to the selectivity samples, five sets of blank and IS blank samples were extracted and analyzed during intra-day assessment. Method selectivity and acceptability of blank matrix samples was evaluated based on a determination of whether a significant interference was found in each analyte's SRM trace. Selectivity and blank matrix samples were considered to be acceptable if no discernible peak at the retention time of each analyte was observed or if the height and area response of a discernible peak was less than 20% of the height and area response of the closest, in run sequence, LLQ standard. For all seven analytes and IS's, no endogenous peaks that would interfere with

Table 3
Intra-day assay accuracy and precision for QC samples ($n = 10$)

Analyte	Mean concentration (ng/mL)	% Accuracy	% CV
(A) Level 1: 3.00 ng/mL for MET, GLY, REP, PIO and ROS. GLI at 6.00 ng/mL and NAT at 15.0 ng/mL			
MET	3.08	103	6.87
GLY	2.93	97.6	5.35
REP	3.03	101	4.76
PIO	2.90	96.8	5.10
ROS	2.98	99.3	5.63
GLI	5.52	92.1	5.52
NAT	13.5	90.0	5.73
(B) Level 2: 150 ng/mL for MET, GLY, REP, PIO and ROS. GLI at 300 ng/mL and NAT at 750 ng/mL			
MET	145	96.4	2.34
GLY	143	95.2	2.94
REP	151	101	0.944
PIO	146	97.2	3.46
ROS	148	98.9	2.83
GLI	275	91.7	2.22
NAT	666	88.8	2.56
(C) Level 3: 375 ng/mL for MET, GLY, REP, PIO and ROS. GLI at 750 ng/mL and NAT at 1880 ng/mL			
MET	343	91.5	2.11
GLY	366	97.7	2.14
REP	374	99.6	1.39
PIO	362	96.5	3.10
ROS	367	97.9	2.16
GLI	701	93.5	1.43
NAT	1690	90.0	2.73

compound quantitation were detected. Evaluation of IS blank plasma samples suggested that no interferences were present from the IS's that could contribute to the analyte *m/z* channels. Extracted-ion chromatograms for all analytes in monkey plasma obtained at the LLQ (for exact LLQ concentrations refer to Table 2) are shown in Fig. 4.

Potential suppression or enhancement of ionization of analytes due to the presence of matrix ions during MS analysis (matrix effect) is a known concern with fast LC–MS/MS systems [32]. Thus, this effect was evaluated with positive ESI–MS and quantitatively reported in terms of the MF (as described previously) for all analytes at three levels of QC concentrations and normalized to the corresponding IS response. The mean value of the MF (IS normalized) was determined across three levels of concentration for each analyte of interest. The resulting MF values ranged from 88.2 to 118% for all seven hypoglycemic drugs. Data suggested that in the present assay no significant matrix effects were observed at the retention times for any of the compounds analyzed in monkey plasma [25–26]. The present analytical method was considered reliable.

3.3. Calibration curves, LLQ and ULQ

Linearity of instrument standard response was determined for each compound with eight different concentration calibration standards. Calibration curves were constructed by plotting individual analyte peak area ratio as normalized to their corresponding IS vs. concentration and fitting these data in regression analysis. Linear regression with a weighing factor of the reciprocal of the concentration squared ($1/x^2$) was applied for all drugs of interest. Intra-day assay accuracy and precision at each analyte's LLQ and ULQ levels based on a five-curve analytical run (standards were prepared in five replicates and analyzed) are shown in Table 2. The mean accuracy for LLQ ranged between 98.7 and 101% and for ULQ 92.1–103%, with the %CV range of 3.31–14.5% and 1.90–3.46%, respectively. All values met the required acceptance criteria as specified previously.

3.4. Accuracy and precision

Table 3 summarizes intra-day accuracy and precision assessments for all the analytes at three levels of QC concentrations. QC samples were prepared at 3.00 (Level 1), 150 (Level 2) and 375 ng/mL (Level 3) for MET, GLY, REP, PIO and ROS; at 6.00, 300 and 750 ng/mL for GLI; and at concentrations of 15.0, 750 and 1880 ng/mL for NAT. The coefficient of variation (%CV) ranged 4.76–6.87%, 0.994–3.46% and 1.39–3.10% for the three levels of QC concentrations, respectively. The overall mean intra-day accuracy of all QC concentrations for all analytes ranged from 88.8 to 103% (Table 3). Inter-day accuracy and precision assessments for all analytes are summarized in Tables 4 and 5. Two QC samples at each concentration level for each analyte were selected from an individual run performed with the 3-day validation assessment in order to determine the inter-day accuracy and precision. Results show that %CV ranged from 2.57 to 10.6% and accuracy ranges from 91.7 to 104%, for all QC samples. Inter-day accuracy and %CV were also determined at the LLQ and are reported in Table 5. All data showed that the method

Table 4
Inter-day assay accuracy and precision for QC samples ($n = 6$)

Analyte	Mean concentration (ng/mL); Level 1	% Accuracy	% CV
MET	2.93	97.7	3.37
GLY	2.97	99.0	4.35
REP	3.04	101	5.28
PIO	2.79	93.0	5.88
ROS	2.90	96.6	7.67
GLI	5.89	98.2	4.39
NAT	13.9	92.7	9.49
Analyte	**Mean concentration (ng/mL); Level 2**	**% Accuracy**	**% CV**
MET	142	94.6	10.2
GLY	146	97.1	4.57
REP	155	104	2.57
PIO	153	102	10.6
ROS	152	101	3.82
GLI	288	95.9	3.10
NAT	693	92.5	3.46
Analyte	**Mean concentration (ng/mL); Level 3**	**% Accuracy**	**% CV**
MET	350	93.3	5.35
GLY	372	99.3	2.71
REP	379	101	2.52
PIO	366	97.6	6.39
ROS	366	97.6	3.53
GLI	709	94.5	2.94
NAT	1720	91.7	3.21

Evaluation was based on a 3-day validation. One set of QCs from each day at each level of concentration was used for calculations. Levels 1, 2 and 3 are defined as in Table 3.

Table 5
Inter-day accuracy and precision at LLQ ($n = 3$)

Analyte name	LLQ			
	Nominal concentration (ng/mL)	Mean concentration (ng/mL)	% Accuracy	% CV
MET	1.00	0.941	94.1	6.32
GLY	1.00	0.996	99.6	2.28
REP	1.00	1.01	101	4.87
PIO	1.00	1.02	102	1.49
ROS	1.00	0.993	99.3	0.201
GLI	2.00	2.04	102	4.95
NAT	5.00	5.27	105	6.48

Table 6
Mean absolute recovery of drugs for Type-2 diabetes extracted from 20 μL of monkey plasma

Analyte	Recovery (%)	% CV
MET	89.7	6.86
GLY	93.8	11.8
REP	94.2	10.2
PIO	88.6	8.55
ROS	89.0	6.75
GLI	86.2	5.09
NAT	93.1	9.20

All values were obtained in triplicate at three levels of QC concentrations. For QC concentrations used for each analyte refer to Table 3.

was acceptable for intra-day and inter-day validation assessments.

3.5. Stability

Stability assessments under different conditions [freeze–thaw (FT) and short-term (ST) room temperature as well as the final matrix (FM) stability] were established for all analytes present in monkey plasma. The analytes were considered stable in biological matrix for FT and ST when ±25% of the reference concentration of the biological matrix stability sample at Day 0 concentration was detected. All compounds were found to be stable at room temperature for at least 5 h (STS) in monkey plasma with mean accuracy range of 90.5–108% and %CV of 0.157–9.34%. All analytes were stable in monkey plasma after three FT cycles with mean accuracy range of 91.4–110% and %CV of 1.05–7.56%. Stability of analytes during FM assessment was considered acceptable when 85–115% of the initial analyte concentration could be detected. All analytes were proven to be stable in the final filtrate solution for at least 24 h at room temperature with accuracy ranging between 87.8 and 105% and %CV ranging between 1.04 and 7.56%. All stability assessments met the acceptance criteria for a valid bioanalytical method.

3.6. Recovery

Table 6 shows the recovery (extraction efficiency) of all seven hypoglycemic drugs from monkey plasma following the protein precipitation extraction procedure. Three concentration levels were tested as specified for the preparation of QC samples. The overall absolute recovery of the analytes ranged from 86.2 to 93.8% with %CV ranging from 5.09 to 11.8%. The data suggest that the extraction efficiency and uniformity met the parameters required for a valid bioanalytical method.

4. Conclusion

The LC–MS/MS method described in this study provided for a fast and reliable multi-drug quantitation approach for seven oral hypoglycemic drugs in a biological matrix. The reported method was shown to be precise and sensitive with an obtainable LLQ range of 1.0–5.0 ng/mL, and validated based on most of the criteria discussed in U.S. Food and Drug Administration Center for Drug Evaluation and Research (FDA/CDER) guidelines for Bioanalytical Method Validation (May, 2001).

Acknowledgment

We would like to thank Helengrace Schuck (Merck & Co., Inc.) for her technical support on this project.

References

[1] G. Valsamakis, S. Kumar, Expert Opin. Pharmacother. 1 (2000) 1413.
[2] C.L. Wu, L.H. Lee, B.R. Lee, C.-J. Lee, Clin. Trials Drugs Biopharm. (2006) 363.
[3] E.S. Horton, C. Clinkingbeard, M. Gatlin, J. Foley, S. Mallows, S. Shen, Diab. Care 23 (2000) 1660.
[4] Y. Hirschberg, A.H. Karara, A.O. Pietri, J.E. McLeod, Diab. Care 23 (2000) 349.
[5] M.S. Rendell, N.B. Glazer, Z. Ye, J. Diab. Complications 17 (2003) 211.
[6] M. Riddle, Am. J. Med. 108 (2000) 15S.
[7] G.E. Dailey, Expert Opin. Pharmacother. 4 (2003) 1417.
[8] S. Bruce, J.-S. Park, F.T. Fiedorek, H.C.S. Howlett, Int. J. Clin. Practice 60 (2006) 783.
[9] J.A. Davidson, S.J. Scheen, H.C.S. Howlett, C.S. Harry, Drug Safety 27 (2004) 1205.
[10] C.J. Bailey, C. Day, Int. J. Clin. Practice 58 (2004) 867.
[11] M. Nakamoto, Diab. Self-Manage. 20 (2003) 14.
[12] The Medical Letter on Drugs and Therapeutics 44 (2002) 107–109.
[13] R.C. Musselman, Home Care Provider 3 (1998) 95.
[14] J.W. Ramsdell, S.N. Braunstein, J.M. Stephens, C.F. Bell, M.F. Botteman, S.T. Devine, PharmacoEconomics 21 (2003) 819.
[15] U.M. Kabadi, M.M. Kabadi, Compr. Ther. 21 (1995) 731.
[16] C.-C. Chou, M.-R. Lee, F.-C. Cheng, D.-Y. Yang, J. Chromatogr. A 1097 (2005) 74.
[17] V. David, C. Barcutean, I. Sora, A. Medvedovici, Revue Roumaine de Chimie 50 (2005) 269.
[18] S. AbuRuz, J. Millership, J. McElnay, J. Chromatogr. B 832 (2006) 202.
[19] N. Koseki, H. Kawashita, N. Miyuki, Y. Nagae, N. Masuda, J. Pharm. Biomed. Anal. 36 (2005) 1063.
[20] Y. Wang, Y. Tang, J. Gu, J.P. Fawcett, X. Bai, J. Chromatogr. B 808 (2004) 215.
[21] K. Heinig, F. Bucheli, J. Pharm. Biomed. Anal. 34 (2004) 1005.
[22] B.-M. Chen, Y.-Z. Liang, F.-Q. Guo, L.-F. Huang, F.-L. Deng, X. Chen, Y.-L. Wang, Anal. Chim. Acta 514 (2004) 185.
[23] S. AbuRuz, J. Millership, J. McElnay, J. Chromatogr. B 817 (2005) 277.
[24] B.L. Kolte, B.B. Raut, A.A. Deo, M.A. Bagool, D.B. Shinde, J. Sep. Sci. 28 (2005) 2076.
[25] G.-P. Zhong, H.-C. Bi, S. Zhou, X. Chen, M. Huang, J. Mass Spectrom. 40 (2005) 1462.
[26] G. Hoizey, D. Lamiable, T. Trenque, A. Robinet, L. Binet, M.L. Kaltenbach, S. Havet, H. Millart, Clin. Chem. 51 (2005) 1666.
[27] Z.J. Lin, D. Desai-Krieger, L. Shum, J. Chromatogr. B 801 (2004) 265.
[28] E. Ho, K. Yiu, T. Wan, B.D. Stewart, K.L. Watkins, J. Chromatogr. B 811 (2004) 65.
[29] H.H. Maurer, C. Kratzsch, T. Kraemer, F.T. Peters, A.A. Weber, J. Chromatogr. B 773 (2002) 63.
[30] C.A. Mueller, W. Weinmann, S. Dresen, A. Schreiber, M. Gergov, Rapid Commun. Mass Spec. 19 (2005) 1332.
[31] W. Zeng, A.L. Fisher, D.G. Musson, A.Q. Wang, J. Chromatogr. B 806 (2004) 177.
[32] L.L. Jessome, D.A. Volmer, LC–GC North Am. 24 (2006) 498.