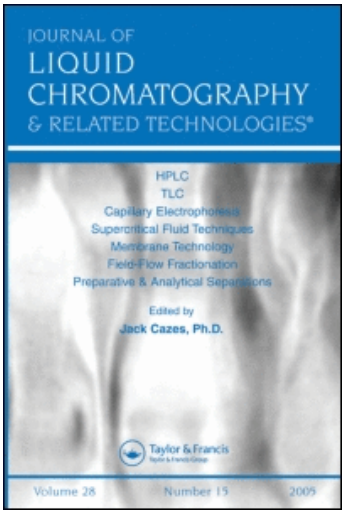

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

LC-MS/MS Determination of Carbamazepine, Pindolol, and Theophylline in Human Serum

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Online publication date: 06 September 2003

To cite this Article Abdel-Hamid, Mohammed E. and Phillips, Oludotun A.(2003) 'LC-MS/MS Determination of Carbamazepine, Pindolol, and Theophylline in Human Serum', Journal of Liquid Chromatography & Related Technologies, 26: 12, 1937 — 1957
To link to this Article: DOI: 10.1081/JLC-120021762
URL: http://dx.doi.org/10.1081/JLC-120021762

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 12, pp. 1937–1957, 2003

LC–MS/MS Determination of Carbamazepine, Pindolol, and Theophylline in Human Serum

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ABSTRACT

A rapid, sensitive, and specific LC–MS/MS method for the determination of carbamazepine (CAZ), pindolol (PIND), and theophylline (THEO) in human serum is presented. The investigated drugs were separated from serum by deproteinization with acetonitrile and were analyzed on an XTerra™ MS, C18, 2.5 μm (2.1 mm × 30 mm) column. Carbamazepine and PIND were eluted with 65% aqueous acetonitrile containing 2 mM ammonium acetate and 0.1% formic acid, whereas THEO was eluted with 65% aqueous acetonitrile containing 2 mM ammonium acetate at flow rate 0.4 mL min^{-1}. The analytes were detected by a triple quadrupole mass spectrometer (Quattro LC, Micromass) using positive (CAZ, PIND) and negative (THEO) electrospray ionization modes. Multiple reaction monitoring (MRM) transitions at 237 > 194, 249 > 116, and 179 > 164 were selected for quantification of CAZ, PIND, and THEO, respectively.

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DOI: 10.1081/JLC-120021762
1082-6076 (Print); 1520-572X (Online)

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Calibration plots were constructed over the concentration ranges 5–50 ng mL^{-1} (CAZ), 10–50 ng mL^{-1} (PIND), and 50–1000 ng mL^{-1} (THEO). The run cycle-time was ~2 min, injection to injection. The assay procedure was highly selective, as no interference either from biological constituents or co-administered drugs was observed. Analysis of control samples of the examined drugs validated the LC–MS/MS method. The overall intra- and inter-assay %CV were in the range 0.9–5.6%, whereas the overall intra- and inter-assay %DEVs ranged −13.6/+7.4%. The protein precipitation method quantitatively recovered the analytes in mixtures from human serum samples in the range of 94.0–104.3%. The data suggest the utility of the developed LC–MS/MS for monitoring serum levels of CAZ, PIND, and THEO in clinical studies.

Key Words: LC-MS/MS; Carbamazepine; Pindolol; Theophylline; Human serum.

INTRODUCTION

Carbamazepine (CAZ), pindolol (PIND), and theophylline (THEO) are widely prescribed in clinical practice as antiepileptic, beta blocking agent and bronchodilator, respectively. Carbamazepine and THEO, in particular, require drug monitoring and individualization of therapy to maintain their target concentrations and to avoid adverse reactions and toxicity. Carbamazepine is frequently administered in generalized tonic–clonic and partial seizures.[1] Its therapeutic concentration range is 4–12 μg mL^{-1}. During drug therapy, CAZ may produce aplastic anemia[2] as a major drug toxicity. Theophylline is a potent bronchodilating agent that exhibits irrigular GI absorption due to low dissolution properties. The therapeutic concentration range of the drug is 5–25 μgmL^{-1}. Theophylline may produce several adverse reactions, which range from GI disturbance to serious arrhythmia and convulsion.[1] Monitoring of serum levels of THEO is important, particularly in infants to avoid adverse reactions. Co-administration of CAZ and THEO enhances the hepatic clearance of THEO.[2] Therefore, a combined drug therapy of CAZ and THEO, necessitates monitoring of drug levels of both drugs to avoid adverse reactions of CAZ and inadequate medication of THEO. Pindolol is a beta-blocking agent, which is used at relatively low doses to control hypertension and cardiac arrhythmias. Pindolol like other beta blocking drugs has a therapeutic level of ~100 ng mL^{-1}. Additionally, PIND has an intrinsic sympathomimetic (agonist) activity,[1] therefore, monitoring of PIND levels is recommended to avoid undesirable effects.

A literature survey on the analytical methodology for determination of CAZ, THEO, and PIND in biological samples indicated that the majority of

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reported methods were based on the application of immunoassays[3,4] and HPLC analyses.[5–12] Although the described procedures were successful, some of the existing methods might suffer from labor-intensive sample preparation and lengthy sample separation, as in HPLC procedures. Furthermore, the HPLC methods require complete resolution of the analytes from co-administered drugs, when combined therapy is prescribed. Recently, the combination of HPLC and ion-trap mass spectrometry (LC–MS) has been proven to be valuable in the analysis of selected antiepileptics and beta blocking agents,[13] sulfur-containing NSAIs,[14] linezolid,[15] and celecoxib.[16] Although, the technique was successful, however, determination of analytes at low concentrations ($<50\,ng\,mL^{-1}$) was not feasible. Recently, tandem mass spectrometry (−MS/MS) using triple quadrupole mass spectrometry has been well-recognized as a potential analytical tool for the analysis of drugs, metabolites, and biomolecules with high degrees of sensitivity and selectivity. The robustness, relatively short-time of analysis, and minimal sample processing have made the technique more attractive in clinical studies. In this presentation, we report on the application of LC–MS/MS for the determination of CAZ, PIND, and THEO in human serum.

The method is based on deproteinization of serum samples using acetonitrile, with subsequent analysis of components using a multiple reaction monitoring (MRM) technique. The validity of the developed method was confirmed by examining the specificity of LC–MS/MS for detecting the analytes in the presence of co-administered antiepileptics and beta blocking agents, and by determining the precision and accuracy of the method for determining the analytes in quality control samples. The applicability of the developed LC–MS/MS for analysis of mixtures of CAZ, PIND, and THEO in human serum was demonstrated.

EXPERIMENTAL

Materials

Carbamazepine, PIND, and THEO were purchased from (Sigma, St. Louis, MO). Human serum was supplied by WINLAB (Laboratory Chemicals/Reagents/Fine Chemicals, UK). HPLC grade acetonitrile (Scharlau Chemie S.A, Barcelona, Spain) was used for the preparation of standards and mobile phases. Other chemicals and reagents used were of analytical grade.

Instrumentation

A triple quadrupole (Quattro LC, Micromass, UK) Mass Spectrometer fitted with a Z-spray ion source was used for all analyses. The mass spectrometer

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was operated in positive (CAZ, PIND) and negative (THEO) electrospray ionization modes, and was coupled to a Waters 2690 Separations Module, Alliance HPLC, and Waters autosampler, USA. Data acquisition and processing were controlled by MassLynx NT 3.5 software.

Mass Spectrometry

Under the HPLC conditions selected, CAZ and PIND form positive $[M+H]^+$ species, whereas THEO forms negative $[M-H]^-$ species. On fragmentation, each parent ion forms a specific product ion that can be used for quantification of the analyte using MRM. The tuning parameters for optimum analysis of the examined compounds and internal standards are listed in Table 1.

Chromatography

After protein precipitation, a 10 μL aliquot of the clear supernatant of CAZ or PIND samples was automatically injected onto a XTerra™ MS, C18, 2.5 μm (2.1 mm × 30 mm) column (Waters) maintained at 25°C, and was chromatographed with 65% aqueous acetonitrile containing 2 mM ammonium acetate and 0.1% formic acid (mobile phase A) and was analyzed using +ESI (Table 1). A 10 μL aliquot of the supernatant of THEO sample was chromatographed with 65% aqueous acetonitrile containing 2 mM ammonium acetate (mobile phase B) and was analyzed using −ESI. The flow rate of the mobile phase in each case was adjusted at 0.4 mL min^{-1}. The run cycle time was ~2 min, injection to injection for all runs.

Table 1. Tuning parameters and MRM transitions of CAZ, CAZEP, PIND, ATEN, and THEO using Quattro LC (Micromass) mass spectrometer.

Compound	Ionization mode	MRM transition	Capillary voltage (kV)	Cone (V)	Collision (eV)
CAZ	+ESI	237 > 194	3.1	30	15
CAZEP (IS)	+ESI	253 > 180	3.1	28	15
PIND	+ESI	249 > 116	3.2	25	20
ATEN (IS)	+ESI	267 > 190	3.2	30	20
THEO	−ESI	179 > 164	3.1	32	20

Source temperature: 100°C
Desolvation temperature: 250°C

Note: CAZ, Carbamazepine; CAZEP, carbamazepine epoxide; PIND, pindolol; ATEN, atenolol; THEO, theophylline; IS, internal standard.

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Standard Solutions

Stock solutions of CAZ, PIND, and THEO were separately prepared by dissolving ~10 mg of each compound in 10 mL acetonitrile to give a drug concentration of 1 μg μL^{-1}. Working solutions were separately prepared by diluting 10 μL aliquots of CAZ and PIND and 100 μL THEO to 10 mL with acetonitrile to give drug concentrations of 1 ng μL^{-1} (CAZ, PIND) and 10 ng μL^{-1} (THEO). The standard solutions were stable in acetonitrile for at least one week at 4°C.

Calibration Curves

Aliquots of CAZ, PIND, and THEO working solutions were separately diluted to 1 mL with control human serum to give calibrators of concentrations 5–50 ng mL^{-1} (CAZ) and 10–50 ng mL^{-1} (PIND) and 50–1000 ng mL^{-1} (THEO). The samples were mixed with the appropriate internal standard and vortexed for 5 min. Aliquots of ~50 μL of the calibrators of each compound were transferred into Eppendorff's tubes and mixed with ~200 μL of acetonitrile. The samples were vortexed for ~2 min and centrifuged for 10 min at 14,000 rpm. An aliquot of ~100 μL of the clean supernatant of each calibrator, was carefully transferred to 150-μL mandrel point insert with poly-spring in 1.5-mL HPLC glass vial. The vials of calibrators of each drug were placed in the carousel in the autosampler and a 10 μL aliquot was automatically injected using the specific MRM transition (Table 1).

Determination of Multiple Reaction Monitoring Transition

The tuning parameters for the determination of CAZ, PIND, or THEO using electrospray MS/MS were optimized by direct infusion of a solution of each compound in the appropriate mobile phase, to the ionization compartment of a mass spectrometer using a Harvard syringe pump at a flow rate 20 μL min^{-1}. The parent ion for each analyte was determined from an MS scan. The high intensity daughter ion was then selected from the MS/MS scan for each compound. An MRM scan was finally selected at the appropriate parent and daughter ions using the optimized cone voltage and collision energy (Table 1).

Sensitivity and Selectivity

Separate human serum samples spiked with CAZ, PIND, and THEO ($n = 3$ of each) at concentrations 1–5 ng/mL (CAZ), 1–10 ng/mL (PIND), and 10–50 ng/mL (THEO) were processed and analyzed as previously described at the specific MRM transitions. The lowest limit of quantitation (LOQ) of each

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compound was determined. The possible interference of biological constituents of human serum and selected co-administered drugs such as antiepileptics (carbamazepine epoxide, clonazepam, diazepam, phenytoin, vigabatrin) and beta blocking agents (acebutolol, atenolol, propranolol) was examined.

Quality Control Samples

Precision and Accuracy

Two sets of samples ($n = 3$) for each compound were prepared in human serum at concentrations 10 and 50 ng mL^{-1} (CAZ, PIND) and 50 and 400 ng mL^{-1} (THEO). One set was kept at room temperature, whereas the other set was kept frozen at -20°C for a week. The samples were treated as mentioned above. The frozen samples were thawed, vortexed, and analyzed as previously described. The values of percentage coefficient of variation (%CV) and percentage deviation from the nominal concentrations (%DEV) were calculated for each compound.

Recovery Studies

Aliquots of CAZ, PIND, and THEO working solutions were spiked to the same serum samples at concentrations 40 ng mL^{-1} for CAZ and PIND, and 200 ng mL^{-1} for THEO ($n = 7$). The samples were processed as previously mentioned and were analyzed twice at positive ESI mode using mobile phase A (CAZ and PIND), and at negative ESI mode using mobile phase B (THEO). The concentrations of analytes in the mixtures were determined from the regression equations representing the calibration curves of CAZ, PIND, and THEO.

RESULTS AND DISCUSSION

Development of Electrospray LC–MS/MS

The electrospray MS/MS conditions for the determination of CAZ, PIND, and THEO in human serum were established. The tuning parameters such as capillary voltage, cone voltage, collision energy, source, and desolvation temperatures were optimized to produce maximum detection of the parent and daughter ions. Based on structural formulas and chromatographic conditions selected, CAZ and PIND were optimally detected using positive ESI, whereas THEO was detected using negative ESI. This might be attributed to the basic and acidic characters of CAZ/PIND and THEO, respectively, which

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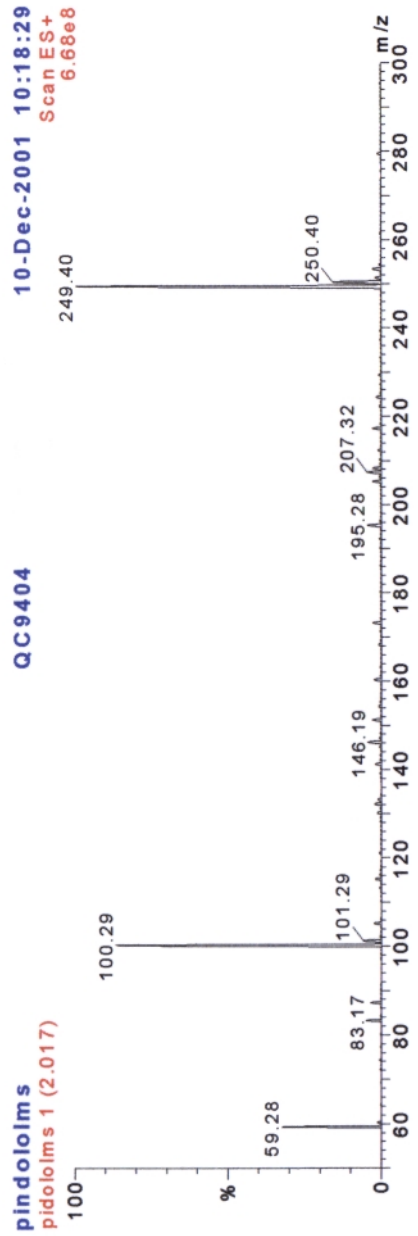

Figure 1. MS spectrum of PIND using positive ESI.

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influences the detection of compounds by ESI. Figure 1 shows the parent ion $[M + H]^+$of PIND at m/z 249. Other parent ions of CAZ, CAZEP (IS), ATEN (IS), and THEO were presented in Table 1. The parent ions were used as the precursor ions in the MS/MS experiments. Representative daughter (MS/MS) scans of PIND and THEO were displayed in Figs. 2 and 3. A schematic diagram (Fig. 4) showing the chemical structures of the parent and daughter ions of CAZ, PIND, and THEO is presented. A MRM scan was selected for the quantitative analysis of CAZ, PIND, and THEO. Using this mode, only a daughter ion, which is related to the target compound is selected, thus minimizing matrix interference due to biological constituents and co-administered drugs.

LC conditions such as the type of the column and composition of the mobile phase were examined for rapid and optimum detection of analytes. An XTerra^{TM} C18, MS column (i.d. 2.1 mm) specific for LC/MS determinations was used at ambient temperature. Two typical mobile phases were used at flow rate 0.4 mL min^{-1}. A mobile phase consisting of 65% aqueous acetonitrile containing 2 mM ammonium acetate and 0.1% formic acid (pH $\sim$ 4.7) was used for analysis of CAZ/CAZEP (IS) and PIND/ATEN (IS). Another mobile phase consisting of 65% aqueous acetonitrile containing 2 mM ammonium acetate (pH $\sim$ 8) was used for analysis of THEO. The high percent content of acetonitrile and the appropriate pH of the mobile phases, permit good detection of the mass ions using positive electrospray ionization (CAZ, PIND) and negative electrospray ionization (THEO). Because tandem mass spectrometry is able to monitor each compound based on its fragmentation properties, it is not necessary to separate the analytes chromatographically so that rapid analysis times and high sensitivity can be achieved. Representative MRM spectra of PIND/ATEN (as IS) and CAZ/CAZEP (as IS) after separation from human serum, are presented in Figs. 5 and 6.

A simple protein precipitation method was applied to permit separation of analytes from serum samples with minimal sample processing. No interference from biological constituents was observed in the chromatograms (Fig. 7). In addition to selectivity of MRM scans, tandem mass spectrometry permits, specifically, detection of the parent ions by using Parent Scan mode. Therefore, the parent ions of beta blocking agents (atenolol, PIND, and propranolol) were selectively detected in the mixtures, since they had the same fragment ion at m/z 116 (Fig. 8).

Calibration Curves and Linearity

Under the selected chromatographic and mass spectrometric conditions, the run cycle time for a complete analysis of CAZ, PIND, and THEO was

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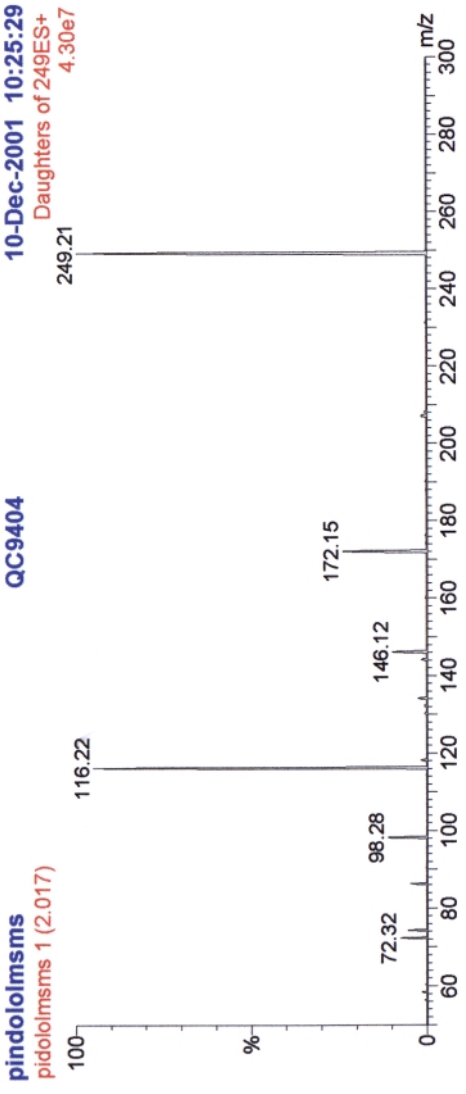

Figure 2. MS/MS spectrum of PIND using positive ESI.

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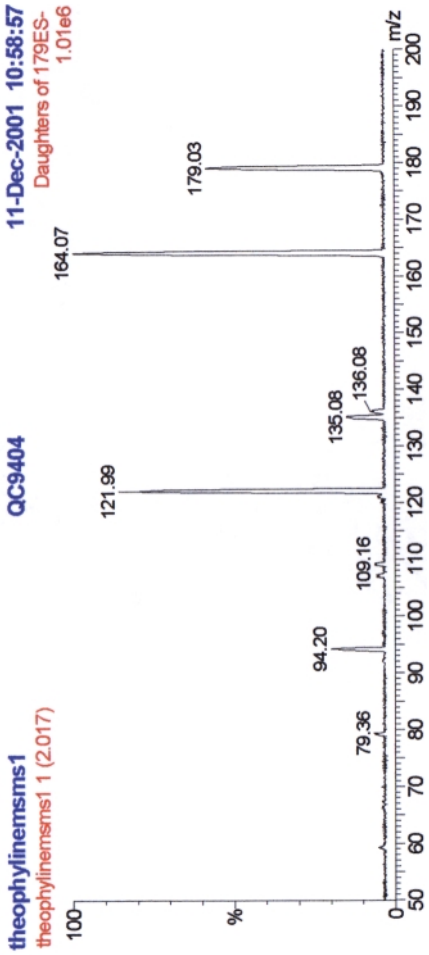

Figure 3. MS/MS spectrum of THEO using negative ESI.

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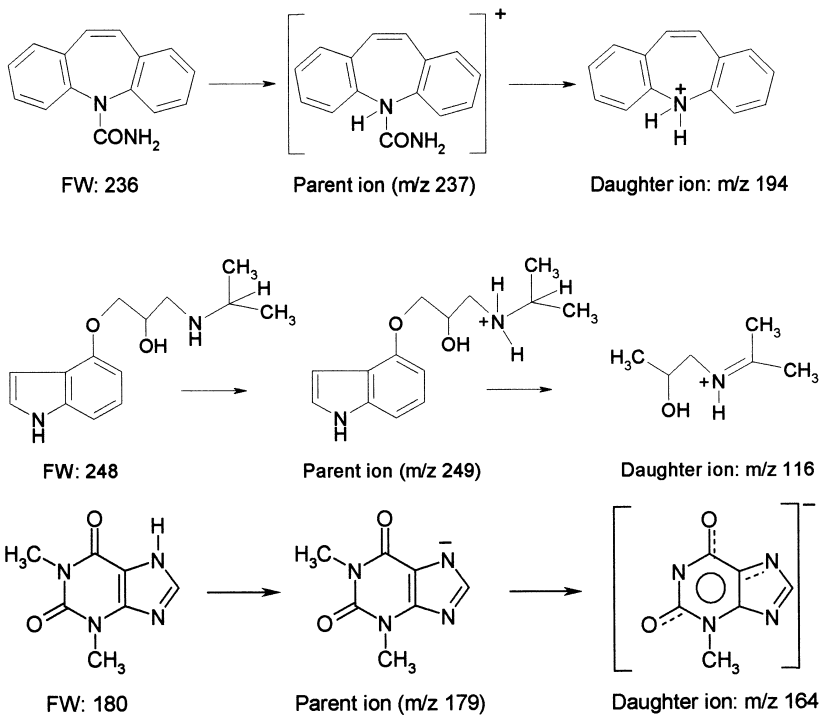

Figure 4. Chemical structures of the parent and product ions of CAZ (FW 236), PIND (FW 248), and THEO (FW 180).

approximately 2 min, injection to injection. Linear relationships of the peak area ratios of CAZ/CAZEP or PIND/ATEN and drug concentration were obtained. In the case of THEO, the calibration curves were described using weighting $(1/x)$ linear regression analysis. Carbamazepine, PIND, and THEO showed linear calibration curves with good regression coefficients ($r > 0.99$). Calibration data were summarized in Table 2.

Sensitivity and Specificity

Under the specified concentration ranges of CAZ, PIND, and THEO, it was not possible to detect and quantify CAZ and THEO using immunoassay, or PIND using HPLC. On the contrary, with the application of tandem mass spectrometry, it was possible to analyze the drugs at the selected

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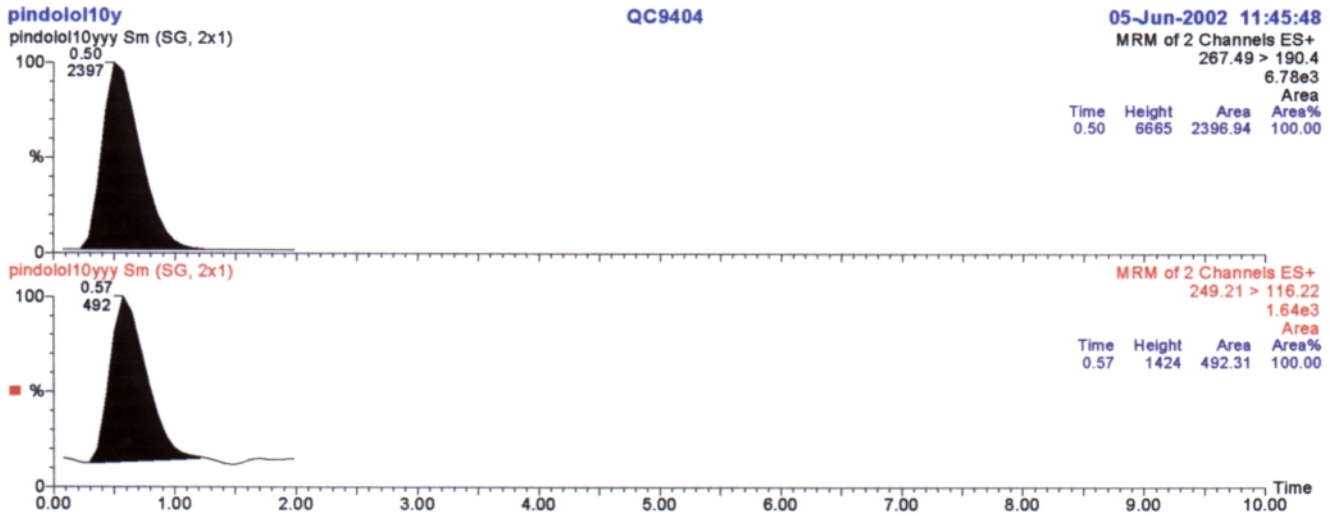

Figure 5. Multiple reaction monitoring chromatograms of PIND (10 ng mL^{-1}) and ATEN (1 μg mL^{-1}) as IS, after separation from human serum.

Figure 6. Multiple reaction monitoring chromatograms of CAZ (10 ng mL^{-1}) and CAZEP (1 μg mL^{-1}) as IS, after separation from human serum.

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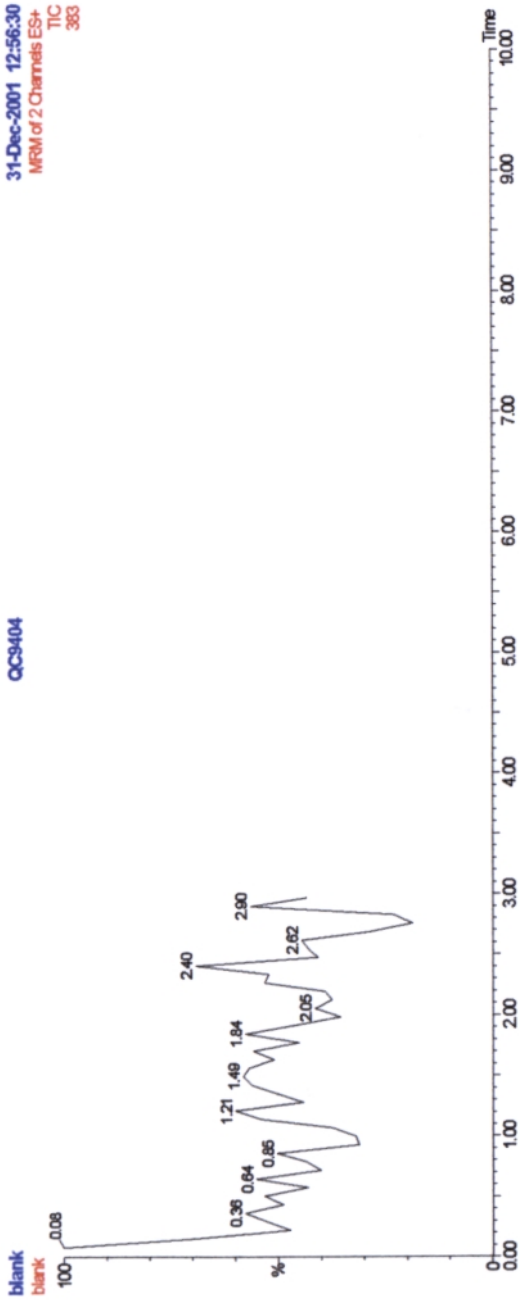

Figure 7. Multiple reaction monitoring scan of drug-free human serum.

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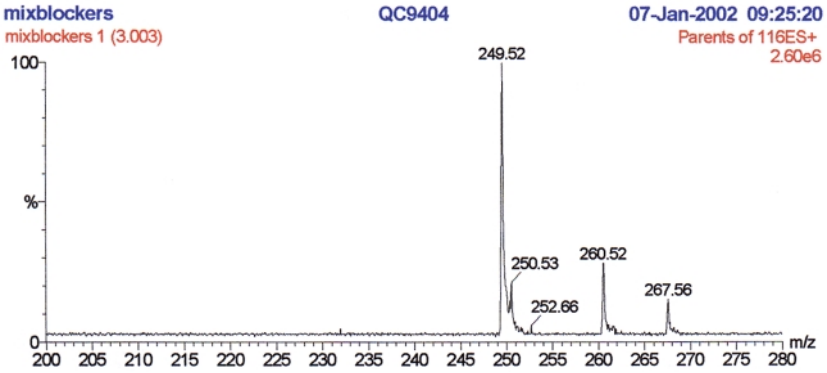

Figure 8. Parent ion scan of atenolol (m/z 267), propranolol (m/z 260), and PIND (m/z 249) in mixture after separation from human serum (daughter ion m/z 116).

concentration ranges using the appropriate electrospray ionization mode. The LOQ was found to be 5, 10, and 50 ng mL^{-1} for CAZ, PIND, and THEO, respectively. The calculated %CV was in the range 2.3–8.6% (Table 2). The specificity of the LC–MS/MS was confirmed by examining the possible interference of some selected co-administered antiepileptics and beta blocking drugs (Table 3). No interference was observed.

Quality Control Samples

Intra-assay precision and accuracy studies of QC samples containing CAZ, PIND, and THEO, revealed that the results were within acceptable levels (%CV 0.9–5.5%) and %DEV −8.9/+7.4% (Table 4). Additionally, the freeze-thaw stability of CAZ, PIND, at concentrations 10 and 50 ng mL^{-1}, and THEO at concentrations 50 and 400 ng mL^{-1} showed that the examined compounds were stable in serum at −20°C for at least one week (%CV 2.2–5.6 and %DEV −13.6/+4.3%) (Table 5).

The applicability of the developed LC–MS/MS to analyze mixtures of the examined drugs in serum was elucidated. The recovery data (Table 6) demonstrated that the developed method was able to detect and quantify the analytes without a need of complete chromatographic separation. Running the system in negative ESI mode, allowed analysis of THEO in the same samples containing CAZ and PIND, which were only detected using +ESI mode. Furthermore, application of +ESI at the MRM transitions

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Table 2. Calibration data for the determination of CAZ, PIND, and THEO in human serum by LC–MS/MS.

Concentration ($ng\,mL^{-1}$)	Peak area (PA)							
CAZ	CAZ			CAZEP (IS)				
5	900	1,200	930	10,500	13,100	9,800		
10	1,525	2,075	1,584	10,726	13,439	10,420		
20	1,725	2,530	2,599	8,152	9,200	10,355		
30	2,094	3,366	3,110	7,025	8,170	7,915		
40	2,496	4,793	4,794	5,635	8,878	10,422		
50	3,181	5,231	5,830	5,685	7,420	10,228		
Using linear regression,								
a	0.011	0.007	0.052					
b	0.011	0.014	0.010					
r	0.9909	0.9985	0.9955					
LOQ	5.0 (%CV: 8.1)							
PIND	PIND				ATEN (IS)			
10	510	595	560	549	1,347	1,507	1,510	1,679
20	686	1,015	944	1,008	1,110	1,663	2,127	1,715
30	948	1,624	1,557	1,685	1,330	1,853	2,301	1,967
40	1,075	2,012	2,076	1,825	1,491	1,777	1,929	1,825
50	1,511	2,644	2,678	2,881	1,070	1,637	1,910	1,916

Using linear regression,				
a	0.0625	0.0366	0.0140	0.0130
b	0.0269	0.0300	0.0300	0.0295
r	0.9987	0.9967	0.9936	0.9994
LOQ	10.0 (%CV: 8.6)			
THEO				
50	316	303	293	300
100	639	630	560	620
200	1,411	1,290	1,175	1,170
400	2,590	2,330	2,040	2,300
600	3,885	4,090	3,190	3,560
1,000	6,330	5,990	5,120	5,430
Using weighting $(1/x)$ linear regression,				
a	39.19	19.22	12.62	3.33
b	5.62	5.68	6.43	6.22
r	0.9966	0.9955	0.9990	0.9915
LOQ	50.0 (%CV: 2.3)			

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Table 3. Multiple reaction monitoring transition of selected antiepileptics and beta blocking agents.

Compound	MRM transition
Carbamazepine epoxide	253 > 180
Clonazepam	316 > 270
Diazepam	285 > 257
Phenytoin	253 > 182
Vigabatrin	130 > 113
Acebutolol	337 > 260
Atenolol	267 > 190
Propranolol	260 > 116

237 > 194 and 249 > 116, permit quantitation of CAZ and PIND, respectively without interference of THEO. The data also confirmed the suitability of the protein precipitation method for separation of CAZ, PIND, and THEO from human serum with minimal sample processing.

CONCLUSIONS

An accurate and highly specific electrospray LC–MS/MS assay for the determination of CAZ and PIND using positive electrospray and THEO using negative electrospray in human serum was developed and validated. The described method offers several advantages such as minimal sample processing, improved sensitivity, distinct short time of analysis, and the ability to

Table 4. Intra-assay precision and accuracy for determination of CAZ, PIND, and THEO in human serum using LC–MS/MS analysis.

Nominal[a] concentration ($ng\,mL^{-1}$)	CAZ		PIND		Nominal[a] concentration ($ng\,mL^{-1}$)	THEO	
	%CV	%DEV	%CV	%DEV		%CV	±%DEV
10	4.4	−1.3 +7.4	4.8	−6.9 +2.4	50	5.5	−8.9 +1.0
50	3.8	−5.9 +1.4	1.4	−4.7 −2.1	400	0.9	+1.2 +2.8

[a]$n = 3$.

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Table 5. Inter-assay precision and accuracy of CAZ, PIND, and THEO quality control serum samples using LC–MS/MS analysis.

Nominal[a] concentration ($ng\,mL^{-1}$)	CAZ		PIND		Nominal[a] concentration ($ng\,mL^{-1}$)	THEO	
	%CV	%DEV	%CV	%DEV		%CV	±%DEV
10	4.4	−12.0	7.6	−9.7	50	5.6	−13.6
		−4.1		+4.3			−3.8
50	3.8	−7.9	3.5	−9.1	400	2.2	−6.4
		−0.8		−2.4			−2.5

[a]$n = 3$.

Table 6. Recovery percentages (%) of CAZ, PIND, and THEO from human serum using LC–MS/MS.

	CAZ 40.0 $ng\,mL^{-1}$		PIND 40.0 $ng\,mL^{-1}$		THEO 200.0 $ng\,mL^{-1}$	
	39.3	98.3	41.4	103.5	207.8	103.9
	37.2	93.0	39.2	98.0	218.8	109.4
	36.3	90.8	37.8	94.5	202.7	101.4
	36.6	91.5	36.6	91.6	210.3	105.2
	38.1	95.3	36.5	91.3	202.0	101.0
	37.4	93.5	38.4	95.9	217.6	108.8
	35.8	89.5	40.6	101.5	205.7	102.9
Mean ± SD	93.1 ± 3.0		96.6 ± 4.7		104.7 ± 3.4	

work with small sample volumes. Further, LC–MS/MS offers an outstanding selectivity to monitor combinations of drugs in serum without prior chromatographic separation. The reported method could be potentially used as an alternative to more traditional techniques such as immunoassays or classical HPLC for monitoring of CAZ, PIND, THEO, and related compounds either in individual or combination therapy.

ACKNOWLEDGMENT

The authors are grateful to the Faculty of Pharmacy, Kuwait University for providing the LC–MS (Quattro LC, Micromass) used for this work.

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Received February 6, 2003
Accepted March 10, 2003
Manuscript 6083

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