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LC/MS determination of the enaminones E139, DM5 and DM27 in rat serum

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Abstract

Enaminones, E139, DM5 and DM27, have been recently recognized as potential anticonvulsant compounds. The molecular masses of these enarminones were proven using ion trap Finnigan mass spectrometer. For conduction of biological studies in animals, a sensitive and selective high-performance liquid chromatography-mass spectrometry (LC/MS) was developed for the determination of the selected enaminones in rat serum. A simple protein precipitation procedure was followed for cleaning up the serum samples before analysis. LC/MS determinations were performed using an APCI probe at 430 °C. Positive ions $(M+1)^+$ were acquired in MS/MS-SRM mode at *m/z* 308.1 (parent *m/z* 340.2) for E139 and *m/z* 262.1 (parent *m/z* 294.1) for DM5. On the other hand, DM27 and E118 (internal standard) were measured in SIM mode at *m/z* 236.5 and 222.5, respectively. Quantitation was based on measurement of the peak area ratio of enaminones (E139, DM5, DM27) and E118 as an internal standard. Calibration curves were linear ($r > 0.9989$) over the concentration range 100–1000 ng ml^{-1} and were free from serum interference. Precision and accuracy studies of control samples showed intra-day and inter-day %RSD < 10.1 and % deviation from nominal concentrations (%DEV) from −4.3 to +10.1. Recoveries of E139, DM5 and DM27 from quality control rat serum samples using protein precipitation method were 92.3, 89.4 and 89.6%, respectively. The reported data suggest the utility of this developed method for structural elucidation and for performing pharmacokinetics studies on the selected enaminones in rats.

Keywords: LC-MS; Enaminones; Rat serum

1. Introduction

Enaminones were recently synthesized as a new and potentially active series of anticonvulsants [1–3]. The anticonvulsant activity of the examined enaminones, E139, DM5 and DM27 (Fig. 1) was evaluated and preliminary studies indicating po-

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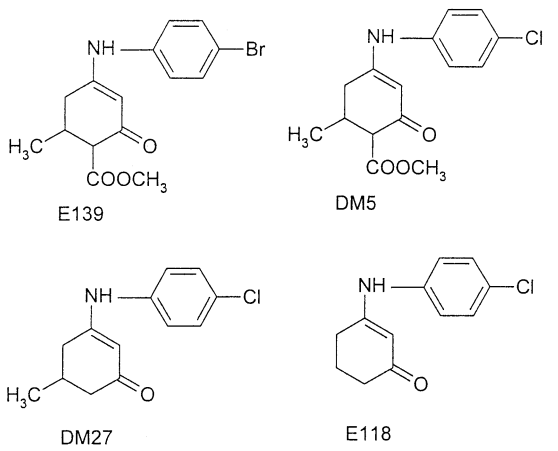

Fig. 1. Chemical structures of enaminones E139, DM5, DM27 and E118.

tent anticonvulsant activity of the compounds encouraged further conduction of pharmacokinetics studies in order to elucidate the absorption, metabolic and excretion profiles in animals. As reported in the literature, an HPLC assay using UV detection was described for the determination of the enaminone DM5 in mouse plasma and brain tissue [4]. Recently, in vitro kinetic stability studies were reported by us using stability-indicating assays to assess the stability of E139 [5] and E118 [6] under simulated physiological acidic and basic conditions. The data indicated relatively higher stability of enaminones in physiological phosphate buffer solution at pH 7.4 compared to 0.1 M hydrochloric acid solution. Generally, the utility of HPLC technique for drug monitoring in biological matrices is always complicated by sample preparation, lengthy sample resolution and lack of identity of the analytes. Recently, LC/MS has been strongly recommended as a powerful tool of elucidating the chemical structure and molecular weight of new medicinal compounds [7] and of quantifying drugs in biological samples [8–10]. This work describes the use of LC/MS technique to confirm the molecular masses of three enaminones namely, E139, DM5 and DM27. Furthermore, the utility of this method for a rapid and accurate determination of the selected enaminones in rat serum using E118 as IS is also reported.

2. Experimental

2.1. Materials and reagents

E139, DM5, DM27 and E118 were chemically synthesized according to the methods of Edafiogho, Scott and co-workers [1–3]. The chemical structures of the prepared enaminones were confirmed using melting point, NMR and elemental analyses (C, H, N, Halogen). The % purity of compounds was at least 99.6%. De-ionized water was used throughout the whole work and was prepared by Mills-Q-System (Millipore Corporation, USA). Rat serum samples were collected via heart puncture of rats after being sacrificed.

2.2. Instrumentation

A Finnigan ion-trap mass spectrometer (LCQ^{TM}) was used for all analyses. The instrument was operated in positive APCI ionization mode and was coupled to Spectra System P 2000 HPLC (Thermo Separations Products) and AS 3000 autosampler (Finnigan Mat). All operational processes and data acquisition were controlled by the instrument LCQ software.

2.2.1. Mass spectrometry

Under the HPLC conditions used, E139, DM5, DM27 and E118 from $(M+H)^+$ species (Fig. 2a–d). On fragmentation, E139 and DM5 produced specific product ions which were used for their specific detection using MS/MS-SRM mode. The parent ions of DM27 and E118 were directly measured using SIM mode. The final MS scanning settings for the analysis of enaminones are shown in Table 1. The APCI probe was used with the following parameters; vaporization temperature 430 °C, capillary temperature 150 °C and corona discharge 4.38 kV.

2.2.2. Chromatography

After protein precipitation, the samples were chromatographed at ambient temperature using Shim Pack CLC-CN C18, 5 μm, 150×4.6 mm^2 column (Shimadzu) and a mobile phase containing acetonitrile and 20 mM ammonium acetate solution (4:1 v/v, pH ~7) at a flow rate of 1 ml min^{-1}.

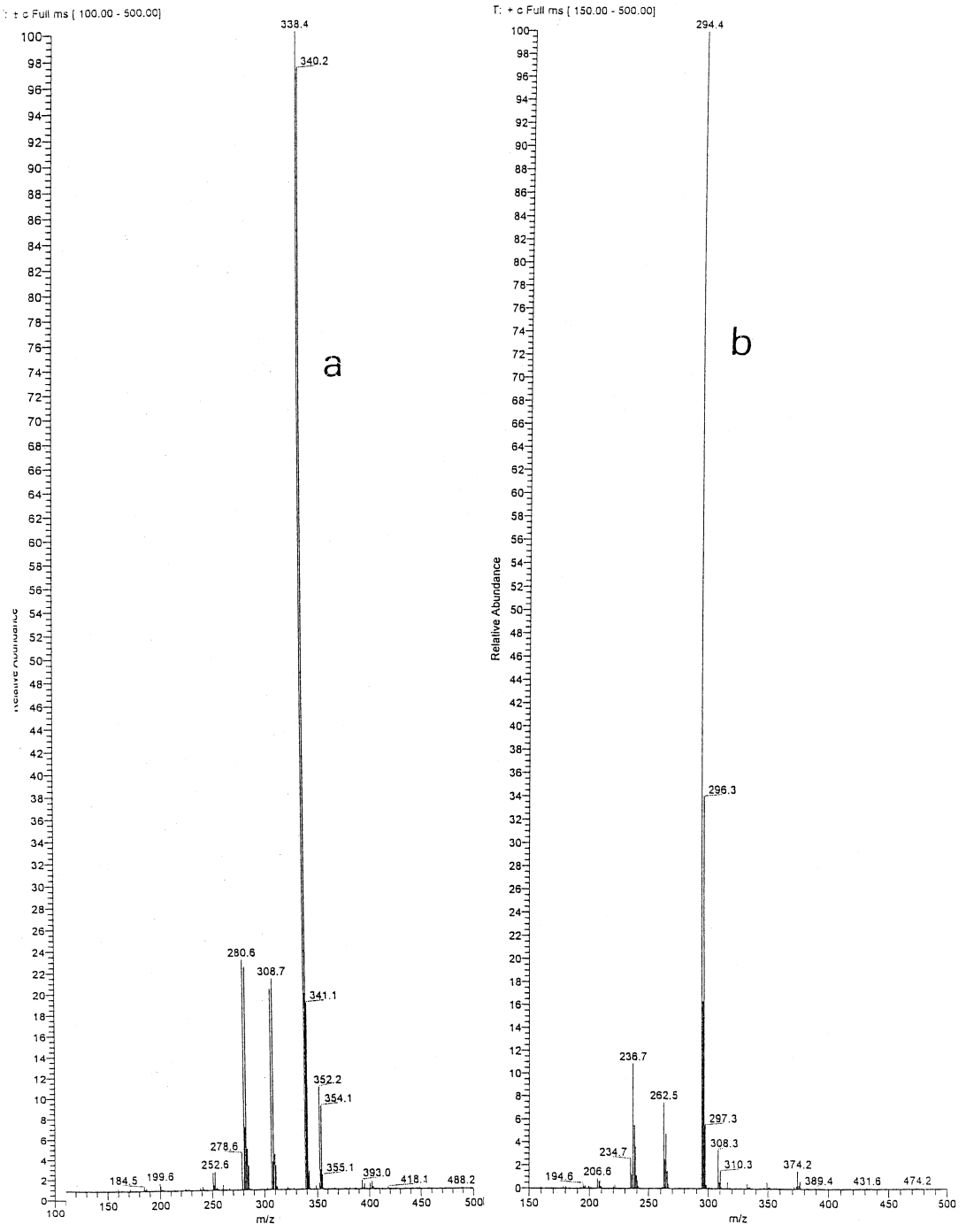

Fig. 2. MS spectra of E139, DM5, DM27 and E118 using APCI probe at 430 °C.

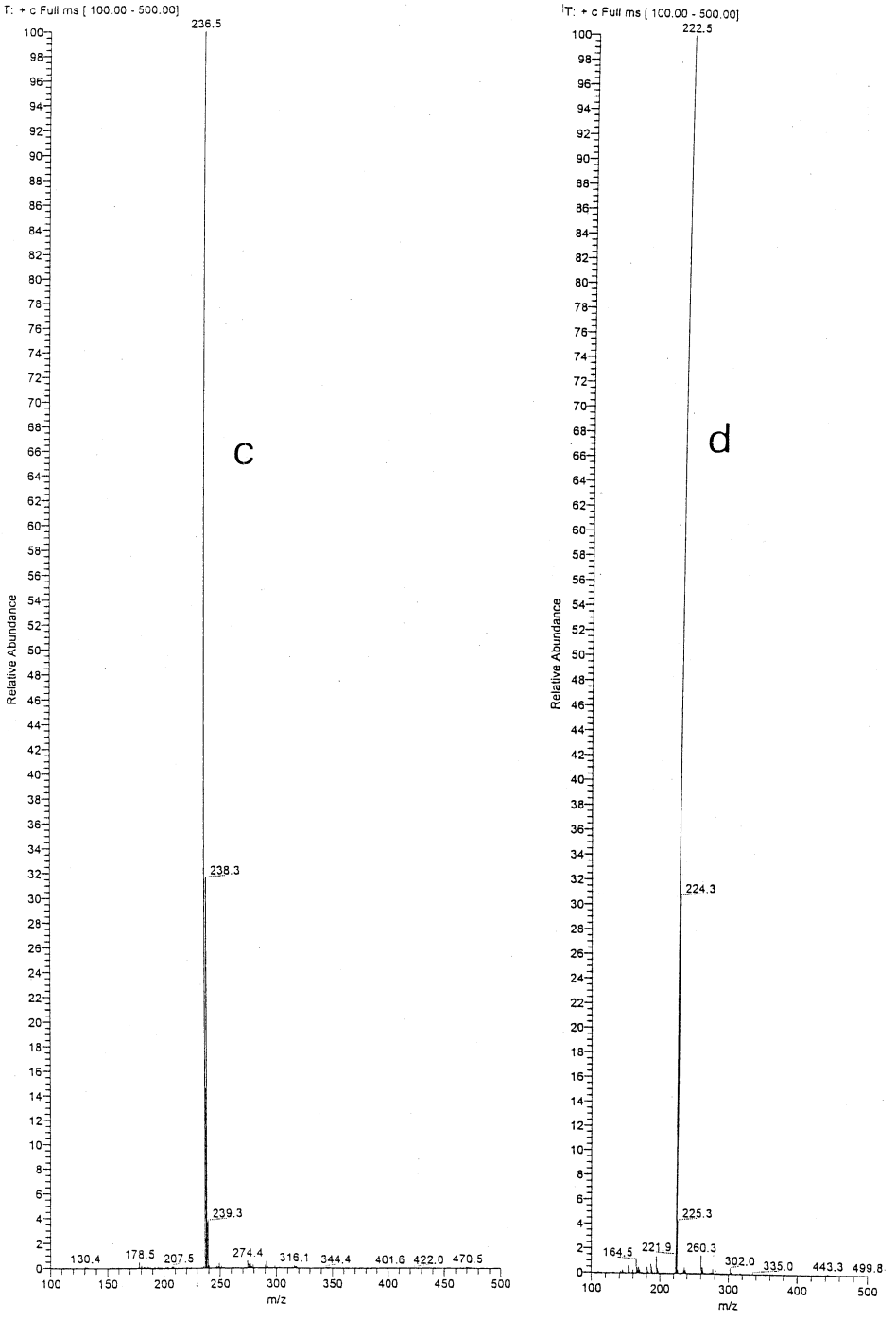

Fig. 2 (*Continued*)

Table 1
Scanning parameters for the analysis of enaminones in rat serum by LC/MS

Enaminone	Scanning mode	Parent ion (m/z)	Fragment ion (m/z)	Collision energy (%)
E139	MS/MS-SRM	340.2	308.1	20
DM5	MS/MS-SRM	294.1	262.1	20
DM27	MS/SIM	236.2	–	–
E118 (IS)	MS/SIM	222.1	–	–

SRM, selected reaction monitoring. SIM, selected ion monitoring.

As a routine, 10 μl samples were either manually injected using 10-μl loop size (Cheminert^{TM}) or automatically injected using acetonitrile/water (1:1) as a needle washing solution.

2.3. Procedures

2.3.1. Standard solutions of enaminones

Stock solutions of E139, DM5 and DM27 were prepared in acetonitrile at concentrations of 1 μg μl^{-1} whereas a stock solution of E118 (IS) was prepared at a concentration of 2 μg μl^{-1} The solutions were stable for ~1 week when stored in glass containers at 4 °C. Working standards of E139, DM5 and DM27 at concentrations 1 ng μl^{-1} and E118 at concentration 2 ng μl^{-1} were used for the preparation of the calibration curves.

2.3.2. Assay procedure

Aliquots of 100 μl rat serum samples were transferred into 1.5 ml polyethylene tubes, spiked with 10, 20, 30, 40 and 100 μl of working solutions of (E139 or DM5 or DM27) and 50 μl of E118 (IS). The solution in each tube was diluted to ~1 ml by adding the appropriate volume of acetonitrile. The samples were vortexed for 1 min and centrifuged at 14000 rpm for 10 min at room temperature. The clear supernatant solutions were transferred into 1.5-ml HPLC glass vials in the autosampler and a volume of 10 μl was automatically injected and analyzed according to a designated LC/MS program.

2.4. Assay validation

2.4.1. Linearity and range

Calibration standards of spiked rat serum samples in a concentration range 100–1000 ng ml^{-1} were prepared and analyzed as above. Three determinations, each of five concentrations (100, 200, 300, 400 and 1000 ng ml^{-1}) were analyzed for each compound. The peak area ratio of an enaminone (E139, DM5, DM27) and E118 (IS) was plotted versus concentration. The slopes, intercepts and regression coefficients of the calibration curves were determined by least squares regression (Table 2). The minimum acceptable coefficient to establish the linearity was 0.99.

2.4.2. Reproducibility and precision

Replicate control rat serum samples ($n = 3$) spiked with E139 or DM5 or DM27 at concentrations specified in Table 3 were prepared and analyzed as above. The concentration of the analyte was determined from the calibration curve. The intra-day %RSD of the assay results were calculated.

2.4.3. Stability studies

Control rat serum samples spiked with E139 or DM5 or DM27 at concentrations specified in Table 3 were prepared and stored at −20 °C for 2 weeks. The samples were thawed on successive days and analyzed as above. The inter-day %RSD of the assay results were calculated (Table 3).

2.5. Accuracy

The accuracy of the developed LC/MS method was determined by comparing the calculated and

Table 2
Calibration curves for the determination of enaminones in rat serum by LC/MS

Enaminone	Concentration range[a] ng ml^{-1}	Regression equation[b]	Regression coefficient (r)
E139	100–1000	PAR = $-0.043+0.0007C$	0.9992
DM5	100–1000	PAR = $0.012+0.0008C$	0.9996
DM27	100–1000	PAR = $0.036+0.0012C$	0.9989

[a] PAR = $a+bC$ (PAR, peak area ratio; a, intercept; b, slope).
[b] Calibration points, 5; mean of three determinations for each.

the normal concentrations of spiked serum samples ($n=3$) at concentrations specified in Table 3. The percent deviation from the nominal concentration (%DEV) was calculated using the following formula: %DEV = [(nominal concentration − calculated concentration)/nominal concentration] × 100.

2.6. Quality control samples

Two control sample sets of (E139 or DM5 or DM27) were prepared at concentrations specified in Table 4. One set was prepared in rat serum and the other set in acetonitrile. The samples were spiked with IS and analyzed as mentioned above. The peak area ratio (PAR) of an enaminone/IS for each concentration for both sets was calculated. The relative percent recovery was determined using the formula: %Recovery = [PAR (serum sample)/PAR (acetonitrile sample)] × 100, whereas, the absolute percent recovery was determined using the formula: %Recovery = [PA (serum sample)/PA (acetonitrile sample)] × 100.

3. Results and discussion

3.1. MS analysis

Fig. 2a–d display the full MS spectra of the enaminones E139, DM5, DM27 and E118 using positive APCI ionization process. Under the selected MS tuning parameters, enaminones exhibited the molecular ions $(M+1)^+$ at m/z 340.2, 294.1, 236.5 and 222.5, respectively. At 20% collision, E139 and DM5 displayed the fragment ions 308.1 and 262.1, respectively. A scheme showing the fragmentation profiles of E139 and DM5 is presented (Fig. 3). On the other hand, the parent ions 236.5 and 222.5 were used to confirm the molecular masses of DM27 and E118, as the MS/MS scans gave no specific fragments at the selected turning conditions.

Table 3
Precision and accuracy data for the determination of enaminones in rat serum by LC/MS

Enaminone	Nominal concentration, ng ml^{-1}	Calculated concentration[a]		%RSD[b]		%DEV[c]	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
E139	100.0	105.2±9.6	106.2±7.8	9.1	7.4	+5.2	+6.2
	400.0	398.4±10.9	390.6±8.4	2.7	2.2	−0.4	−2.4
DM5	100.0	99.2±6.4	95.7±9.2	6.5	9.6	−0.8	−4.3
	1000.0	990.4±55.3	1030.1±45.8	5.6	4.5	−0.9	+3.1
DM27	100.0	104.1±6.9	110.1±7.1	6.6	6.4	+4.1	+10.1
	1000.0	986.1±31.1	1098.2±25.2	3.2	2.3	−1.4	+9.8

[a] Mean of three determinations.
[b] %RSD, % relative standard deviation.
[c] %DEV, % deviation from nominal value.

Table 4
Relative and absolute recoveries of E139, DM5 and DM27 as determined by LC/MS in quality control samples

Enaminone[a]	Concentration, ng ml^{-1}	PAR[b]		Relative recovery, %	Absolute recovery %	
		Serum	Acetonitrile		Enaminone	IS[c]
E139	100	0.042	0.043	94.9	95.0	96.7
	200	0.101	0.118	85.6	78.3	91.0
	400	0.244	0.253	96.4	96.3	99.9
Mean, %				92.3	89.9	95.9
DM5	100	0.076	0.091	83.5	77.9	92.7
	300	0.246	0.282	87.2	79.1	90.9
	1000	0.827	0.849	97.4	95.6	98.4
Mean, %				89.4	84.2	94.0
DM27	100	0.152	0.172	88.4	80.2	90.4
	500	0.650	0.731	88.9	83.4	95.1
	1000	1.283	1.401	91.6	87.8	93.8
Mean, %				89.6	83.8	93.1

[a] Mean of three determinations.
[b] Peak area ratio of an enaminone/IS.
[c] Internal standard.

3.2. Development of LC/MS methods

The fragment ions of E139 and DM5 in MS/MS scans and the parent ions of DM27 and E118 (IS) in MS scans were utilized to determine the examined enaminones in rat serum by HPLC/MS analyses. To improve the selectivity of the method, SRM scans at m/z 308.1 and 262.1 were selected to determine E139 and DM5, respectively, whereas SIM scans at m/z 236.5 and 222.5 were utilized to determine DM27 and E118 under the selected chromatographic conditions. The composition of the mobile phase was varied to optimize MS detection of the parent and fragment ions of enaminones under the selected APCI tuning parameters. It was found that a mobile phase comprising of acetonitrile and 20 nM ammonium acetate solution in a ratio 4:1 v/v (pH $\sim$7) was appropriate. Due to high sensitivity of the developed LC/MS, small volumes (100 μl) of the spiked rat serum samples were used to perform the analysis. Also, due to high selectivity, it was possible to identify and quantify each component without a need of complete resolution of enaminones and internal standard as in the classical HPLC technique. A simple protein precipitation rather than, solvent or solid-phase extraction methods, was adopted to clean up the serum samples before injection. As shown in Figs. 4–6, E139 or DM5 or DM27 and E118 (IS) were extracted from the total ion chromatogram of samples without complete column separation. The assay run-time was <3.5 min. Enaminone-free rat serum samples exhibited negligible interferences under the selected LC/MS conditions (Fig. 7).

3.3. Validation of LC/MS assay

The calibration curves for E139, DM5 and DM27 showed linearity over the concentration range of 100–1000 ng ml^{-1} with consistent intercepts, slopes and excellent regression coefficients ($r > 0.9989$) (Table 2). The limit of quantitation for E139, DM5 and DM27 was 100 ng ml^{-1}. The selected concentrations were within the expected rat serum levels following intra-peritoneal injection of solutions of enaminones, as reported by Cox and co-workers [4]. The precision of the developed LC/MS method was evaluated by analyzing control rat serum samples, by determining the intra-day %RSD for E139, DM5 and DM27 at various concentration levels (Table 3). Furthermore, the stability of E139, DM5 and DM27 in control and frozen rat serum samples

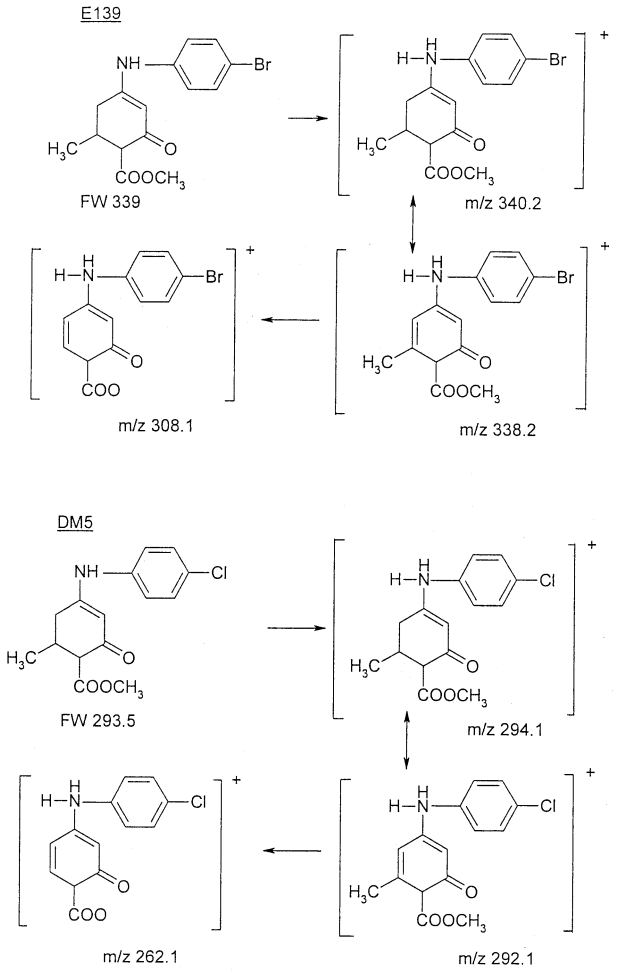

Fig. 3. APCI-MS/MS fragmentation patterns of E139 and DM5.

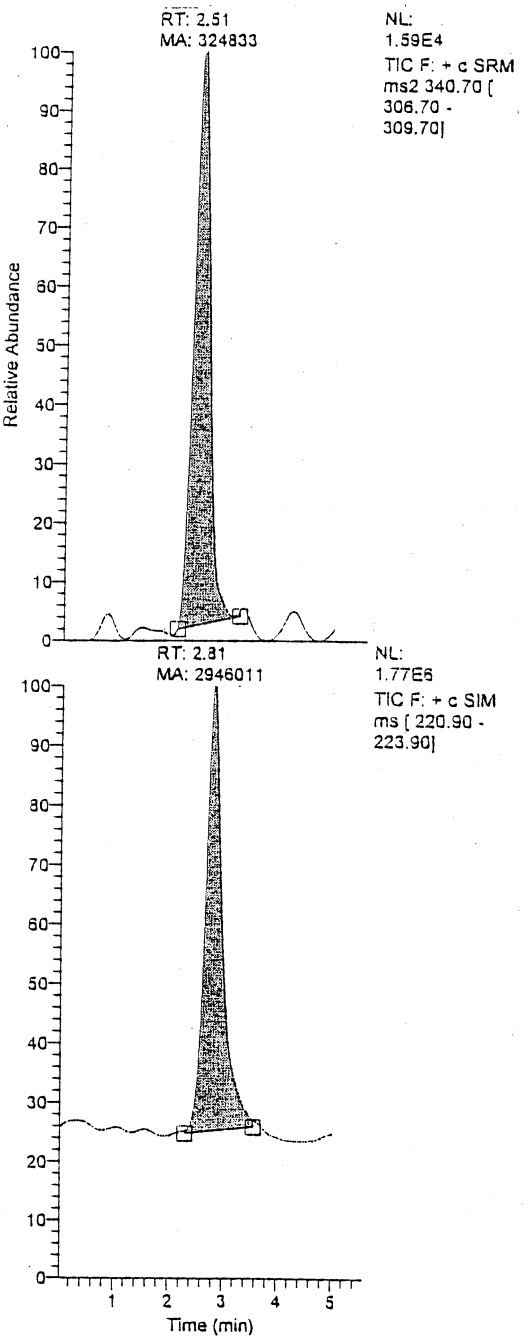

Fig. 4. LC/MS chromatograms of E139 and E118 (IS) in rat serum (C: 200 ng ml^{-1})

was studied by determining the inter-day %RSD of the enaminones concentrations after thawing the samples using LC/MS method. As shown in Table 3, the %RSD intra-day was in the range of 2.7–9.1%, whereas the %RSD inter-day was in the range of 2.2–9.6%. Furthermore, the accuracy of the developed LC/MS assay was evaluated by determining the intra-day and inter-day percent deviations (%DEV) from the nominal concentrations. These values ranged from −4.3 to +10.1%.

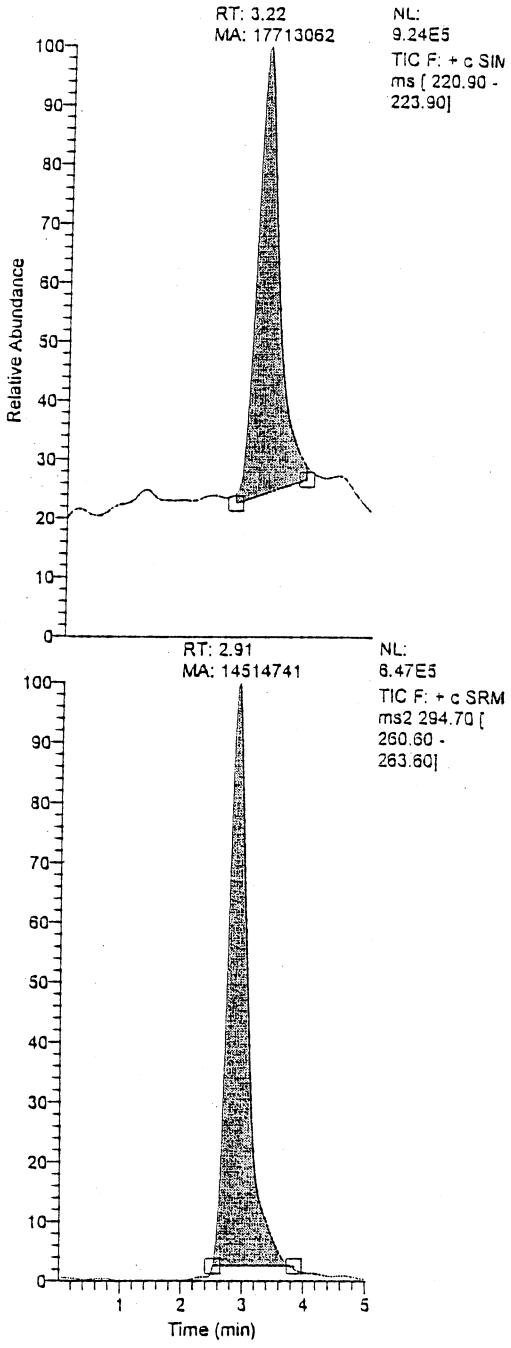

Fig. 5. LC/MS chromatograms of DM5 and E118 (IS) in rat serum (C: 1000 ng ml^{-1}).

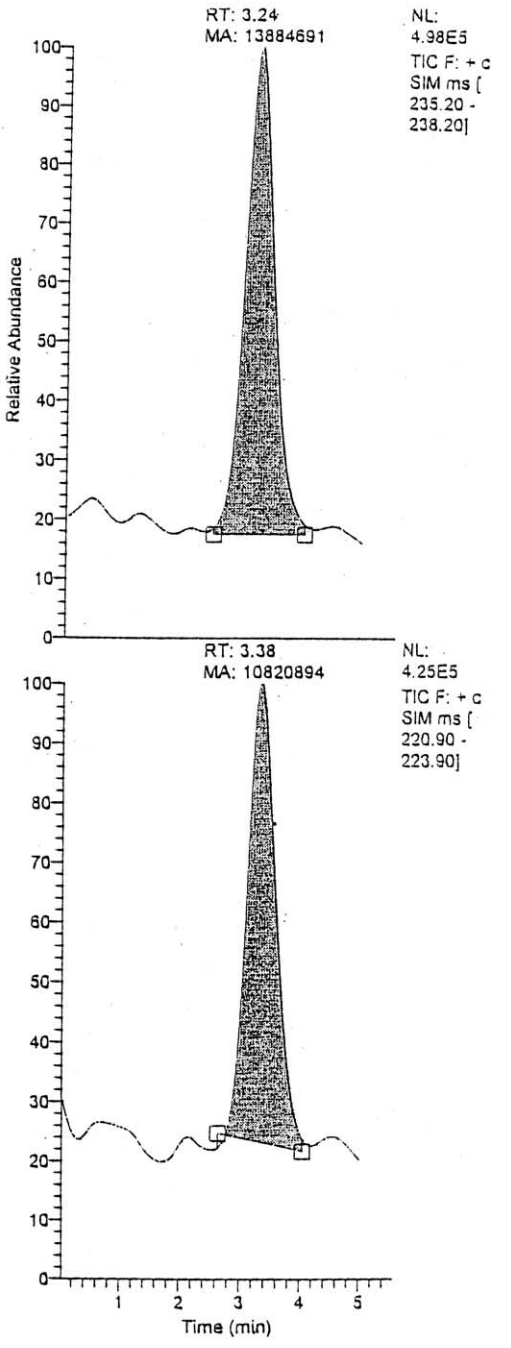

Fig. 6. LC/MS chromatograms of DM27 and E118 (IS) in rat serum (C: 1000 ng ml^{-1}).

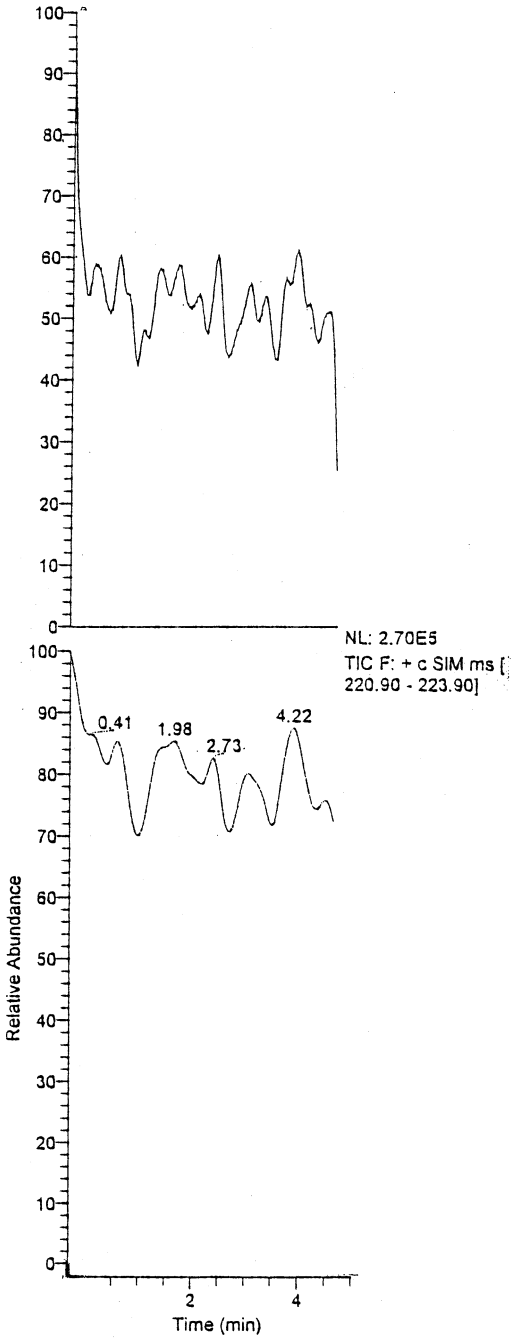

Fig. 7. LC chromatogram of rat serum control.

3.4. Quality control samples

Quality control samples spiked with the enaminones E139 or DM5 or DM27 at concentrations mimicking biological samples were analyzed using the developed method. Relative and absolute recoveries were calculated to evaluate the protein precipitation method for the preparation of rat serum samples for analysis by LC/MS. As shown in Table 4, relative recoveries of 92.3, 89.4, 89.6% were determined for E139, DM5 and DM27, respectively. Absolute recoveries of 89.9, 84.2, 83.8% for enaminones and 94.3% for IS, were calculated. The derived data confirmed that the developed LC/MS method was appropriate for the analysis of enaminones (E139, DM5 and DM27) in rat serum using protein precipitation method that ensured mineral sample processing.

4. Conclusion

The described LC/MS method has a high potential for structure elucidation, identify and quantification of enaminones in biological samples, MS and MS/MS spectra permit identification and characterization of E139, DM5 and DM27, whereas MS/MS-SRM or MS-SIM permits quantification of enaminones in nanogram range. Validation results confirmed the precision and accuracy of the developed method for the analysis of the selected enaminones in rat serum at relatively low concentrations. Furthermore, the results indicated that the enaminones are stable in spiked serum samples for at least 2 weeks when stored at -20 °C. Quality control data proved the specificity of LC/MS method for monitoring enaminones levels in rat serum at concentrations that mimic biological samples using protein precipitation method. The run-cycle time for sample analysis was <3.5 min with minimal sample processing. These advantages support the utility of this developed method for planned pharmacokinetics and toxicological studies of enaminones in biological samples.

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