Determination of Paracetamol and Tramadol Hydrochloride in Pharmaceutical Mixture Using HPLC and GC–MS

Tarek Belal[1], Tamer Awad[2], and **C. Randall Clark[2]***

[1]Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Alexandria University, Alexandria 21521, Egypt and [2]Department of Pharmacal Sciences, Harrison School of Pharmacy, Auburn University, Auburn, AL 36849, USA

Abstract

Two simple, rapid, and selective analytical procedures were developed for the simultaneous determination of paracetamol (PR) and tramadol hydrochloride (TR) in a binary mixture using high-performance liquid chromatography with UV detection (HPLC–UV) and gas chromatography with mass spectrometry (GC–MS) techniques. HPLC resolved the two compounds on a Hypurity Advance column using a mobile phase consisting of phosphate buffer pH 6.3 and acetonitrile (90:10, v/v). PR and TR were detected by their UV absorption at 220 nm. GC–MS involved separation of the two compounds using 100% dimethylpolysiloxane (Rtx-1) column with temperature programming. The EI mass spectrum of PR was characterized by $[M]^+$ at 151 and a base peak at *m/z* 109 while TR mass spectrum was characterized by $[M]^+$ at 263 and a base peak at *m/z* 58. Quantification of the analytes in both methods was based on measuring the peak areas. The reliability and analytical performance of the proposed methods including linearity, ranges, precision, accuracy, detection, and quantification limits were statistically validated. Calibration curves were linear over the range 10–400 µg/mL for both PR and TR using the HPLC method and over the ranges of 75–500 and 25–350 µg/mL for PR and TR, respectively, using the GC–MS method. The proposed methods were successfully applied for the determination of the two compounds in laboratory-prepared mixtures and in commercially available tablet formulation. No interference peaks were observed from common pharmaceutical adjuvants. The results compared favorably with those obtained by a derivative spectrophotometric method.

Introduction

Paracetamol (PR) or acetaminophen, *N*-(4-hydroxyphenyl)-acetamide, is one of the most popular and widely used drugs for the treatment of pain and fever. It occupies a unique position among analgesic drugs. According to a recent update of the American College of Rheumatology (ACR) guidelines for osteoarthrosis, PR remains a first-line therapy because of its cost, efficacy, and safety profiles (1). Unlike non-steroidal anti-inflammatory drugs, it is considered to have no anti-inflammatory activity and does not produce gastrointestinal damage (1,2). Unlike opiates it is almost ineffective in intense pain and has no depressant effect on respiration (1,2). The major advantage of PR lies in its relative lack of serious side effects (1,2). Although PR has been used clinically for more than a century, its mode of action has been a mystery until recently when it was discovered that the analgesic effect of PR is due to the indirect activation on cannabinoid CB1 receptors (1,2). Tramadol hydrochloride (TR), (1RS,2RS)-2-[(Dimethyl-amino)methyl]-1-(3-methoxy-phenyl) cyclohexanol hydrochloride, is a centrally acting analgesic consisting of two enantiomers, both of which contribute to the analgesic activity via different mechanisms (3). (+)-Tramadol is an agonist of the µ-opioid receptor, and it inhibits serotonin reuptake whereas (–)-tramadol inhibits norepinephrine reuptake, enhancing inhibitory effects on pain transmission in the spinal cord (3). TR is an effective and well-tolerated agent that reduces pain resulting from trauma, renal or biliary colic, and labor, and also for the management of chronic pain of malignant or nonmalignant origin, particularly neuropathic pain (3). TR appears to produce less constipation and dependence than equi-analgesic doses of strong opioids (3). The analgesic efficacy of tramadol can further be improved by combination with a non-opioid analgesic (3). Structures of PR and TR are shown in Figure 1.

The United States Pharmacopeia suggests a spectrophotometric A_{max} procedure for the analysis of PR powder and high-performance liquid chromatography (HPLC) for all its preparations (4). The British Pharmacopoeia shows various analytical procedures for the assay of PR in bulk powder and dosage

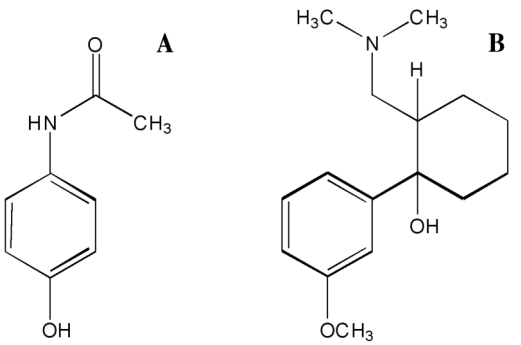

Figure 1. Structures of paracetamol (A) and tramadol (B).

* Author to whom correspondence should be sent: Email: clarkcr@auburn.edu.

forms including cerium sulphate titrimetry, A_{max} spectrophotometry, and HPLC. For TR, potentiometric non-aqueous titration is applied for the assay of the bulk powder whereas HPLC is used for the capsules (5).

Several analytical procedures have been reported for the determination of the two compounds. Espinosa Bosch et al. (6) in their review article demonstrated over 300 reports of different optical, electrochemical, and chromatographic techniques used for quantification of PR in pharmaceutical formulations and biological samples in the last four decades. TR was determined in different matrices using a variety of analytical techniques including HPLC (7,8), gas chromatography with mass spectrometry (GC–MS) (9,10), thin layer chromatography (TLC)–densitometry (11), capillary electrophoresis (12,13), adsorptive stripping voltammetry (14), square-wave voltammetry and flow injection analysis system with amperometric detection (15), selective PVC membrane electrodes (16), spectrofluorimetry (17), and spectrophotometry (17,18).

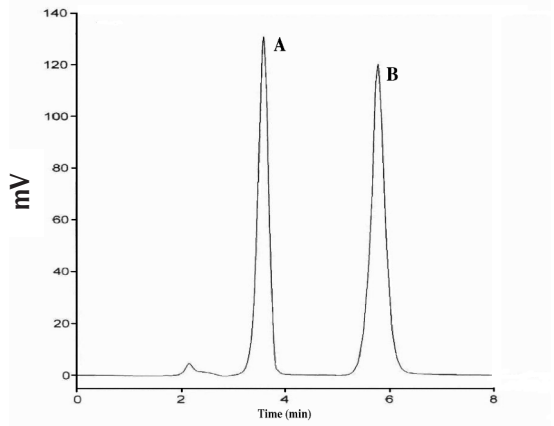

Figure 2. HPLC chromatogram of 20-µL injection of mixture of 50 µg/mL PR (A) and 100 µg/mL TR (B).

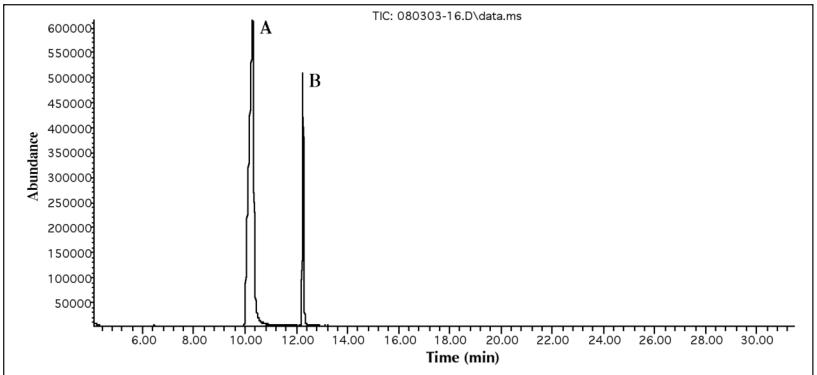

Figure 3. GC chromatogram of 1-µL injection of mixture of 400 µg/mL PR (A) and 50 µg/mL TR (B).

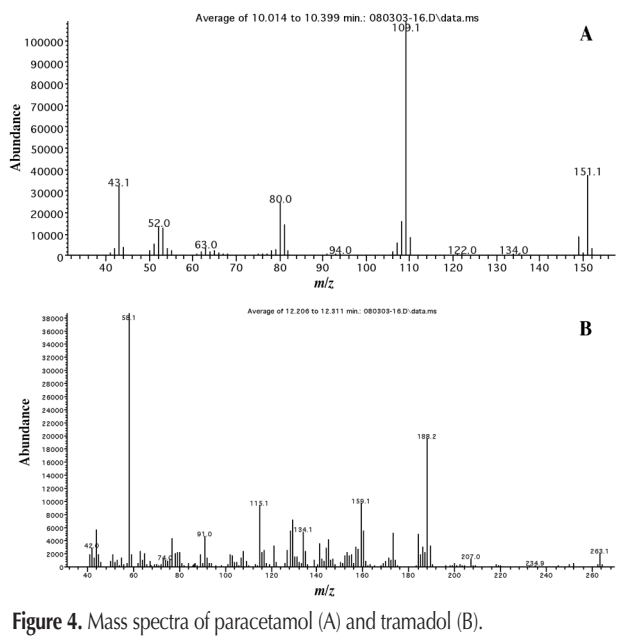

Figure 4. Mass spectra of paracetamol (A) and tramadol (B).

The simultaneous determination of the two drugs has been reported in a few publications. They were estimated in human plasma samples using liquid chromatography (LC)–MS (19,20). In tablets, they were determined using spectrophotometric (21,22) and reverse-phase HPLC methods using C_{18} columns (23–25). No attempts have yet been made to determine this drug mixture by GC. In the present work, two chromatographic procedures are proposed and validated for the simultaneous determination of PR and TR in their bulk form and in tablet form. The HPLC method involved the use of different stationary phase other than the traditional C_{18} column and a different mobile phase composition. The second method involved the application of GC–MS.

Experimental

Instrumentation

The HPLC system comprised of an LC-10AS Shimadzu liquid chromatograph (Kyoto, Japan) with SIL-10A auto-injector and SPD-10AV UV-visible detector. EZstart 7.4 chromatography software (Shimadzu) was used for processing of data and peaks integration. The column used was Hypurity Advance column (5 µm, 150 × 4.6 mm i.d., Thermo-Hypersil Keystone, Bellefonte, PA). The flow rate was 1.0 mL/min, and the injection volume was 20 µL. The detector was set at λ = 220 nm, and all determinations were performed at room temperature.

The GC–MS study was conducted using an Agilent Technologies (Santa Clara, CA) 7890A gas chromatograph and an Agilent 7683B auto injector coupled with a 5975C VL Agilent mass selective detector. The injection volume was 1 µL, and the mass spectral scan rate was 2.86 scans per second. The GC was operated in splitless mode with a carrier gas (helium grade 5), flow rate at 0.7 mL/min, and a column head pressure of 10 psi. The mass spectrometer was operated on the electron impact (EI) mode using an ionization voltage of 70 eV and a source temperature of 230°C. The GC injector was maintained at 250°C and the transfer line at 280°C. The temperature program used consisted of an initial temperature hold at 70°C for 1 min, ramped up to 250°C at a rate of 30°C/min followed by a hold at 250°C for 20 min. The mass spectra reported were obtained by background subtraction and are the average of at least five scans. The chromatographic separations (and collection of retention data) were carried out on

a 30 m × 0.25 mm-i.d. column coated with 0.25 µm 100% dimethyl polysiloxane (Rtx-1) purchased from Restek Corporation (Bellefonte, PA).

Drugs and reagents

PR (minimum 99.0%) (Sigma Aldrich, St. Louis, MO) and TR hydrochloride (≥ 99.0%, HPLC-grade) (Fluka BioChemika, Buchs, Switzerland) were used in the study. Tramol-Plus tablets (Zypher, Laborate Pharmaceuticals India Ltd, India, B.N. ZTPT 702) labeled to contain 50 mg TR and 325 mg PR per tablets were purchased from a local commercial source. HPLC-grade solvents including methanol and acetonitrile (Fisher Scientific, Fair Lawn, NJ), HPLC-grade *o*-phosphoric acid 85% (Fisher Scientific, Fair Lawn, NJ), and high purity distilled water were used in the study.

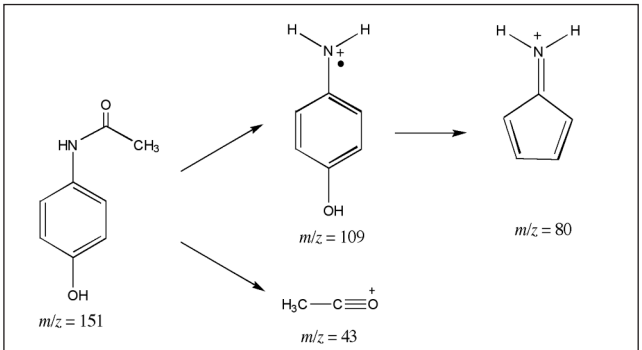

Figure 5. Fragmentation pattern of paracetamol.

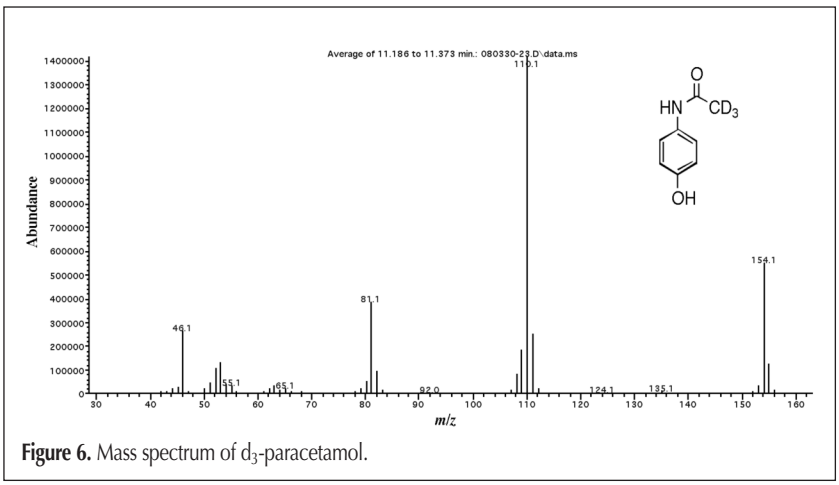

Figure 6. Mass spectrum of d_3-paracetamol.

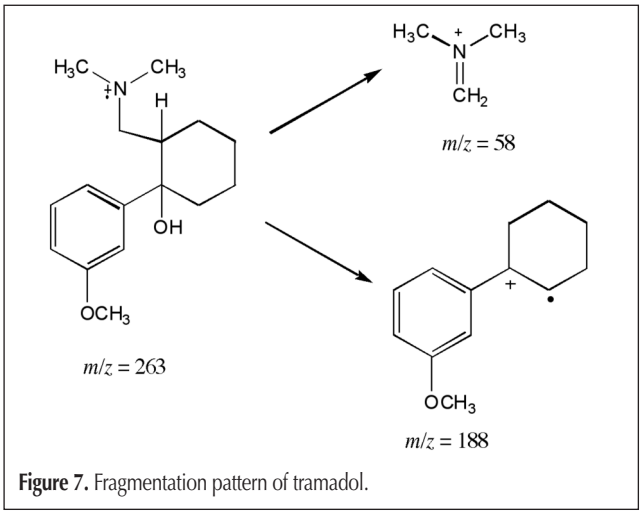

Figure 7. Fragmentation pattern of tramadol.

General procedures

HPLC

Phosphate buffer was prepared by mixing aqueous 0.05 M phosphoric acid solution with 0.2 M sodium hydroxide solution to reach pH 6.3. The mobile phase was prepared by mixing phosphate buffer pH 6.3 and acetonitrile in the ratio of 90:10 (v/v), then it was filtered and degassed.

PR and TR stock solutions (500 µg/mL) were prepared in methanol. The working solutions were prepared by dilution of the stock solutions with the mobile phase to reach a concentration range 10–400 µg/mL for both PR and TR. Injections were made for each concentration and chromatographed under the previously described LC conditions.

GC–MS

PR and TR stock solutions (1000 µg/mL) were prepared in methanol. The working solutions were prepared by dilution of the stock solutions with methanol to reach concentration ranges 75–500 and 25–350 µg/mL for PR and TR, respectively. Injections were made for each concentration and chromatographed under the previously described GC conditions.

The peak areas obtained from both HPLC and GC methods were plotted against the corresponding concentrations to obtain the calibration graphs.

Assay of tablets

A total of 10 tablets were weighed and finely powdered. Methanol (60 mL) was added to a quantity of the powdered tablets equivalent to 260 mg PR and 40 mg TR, stirred for 10 min, then filtered into a 100-mL calibrated flask. The residue was washed with 2 × 10 mL methanol, and washings were added to the filtrate and diluted to final volume with methanol. Aliquots of the tablet solution (prepared in methanol) were diluted with either the HPLC mobile phase (for HPLC measurement) or methanol (for GC measurement) to obtain final concentrations within the previously mentioned ranges and then treated as under the procedures for HPLC and GC–MS methods.

Results and Discussion

HPLC

A liquid chromatography method coupled with UV detection was developed to provide a suitable procedure for the rapid and reliable quality control analysis of PR and TR in their combined pharmaceutical preparation. Several reverse-phase stationary phases were tried including C_{18}, C_8, and phenylhexyl columns. Although these columns gave satisfactory resolution of the two analytes, PR eluted at the void volume, regardless of the composi-

tion of the mobile phases. This can be attributed to the weak retention of the relatively polar PR on these non-polar stationary phases. The Hypurity advance column with its embedded polar character, which contains a polar amide group embedded within a C_8 chain, gave better retention for PR with good resolution of the two analytes. Consequently, it became the column of choice for this study.

Several mobile phases were tried using various proportions of several solvents and buffers at different pH values. The best resolution and analysis time was obtained through isocratic elution using a mobile phase consisting of 10% (by volume) acetonitrile in phosphate buffer pH 6.3 (Figure 2). Methanol produced broad asymmetric peak with TR, hence it was not used as a component of the mobile phase. Buffer pH was evaluated in the range from 2.0 to 7.0, and best resolution and peak shapes were achieved at pH ranging from 6.0 to 6.5. Lower acidic pH values led to weak resolution whereas increasing pH to more than 6.5 resulted in tailing and decrease in the sharpness of the peaks. Quantification was made with UV detection based on measuring the peak area. The UV detector was set at 220 nm, which was found to be optimum in measuring the two analytes.

The previously described chromatographic conditions showed symmetric peaks and adequate resolution (R_s = 4.67) between PR (t_R = 3.65 ± 0.026 min) and TR (t_R = 5.81 ± 0.043 min) as shown in Figure 2. The capacity factors (k') were found to be equal to 0.68 and 1.70 for PR and TR, respectively, and the selectivity (α) was 2.50.

GC–MS

The second part of this study involved the determination of PR and TR in a binary mixture using GC–MS. Previous studies have shown the GC–MS determination of PR and TR individually in different matrices (9,10,26). However, the simultaneous determination of these two analytes in pharmaceutical binary mixture using GC–MS has not been previously reported. The primary goal of this part of the study is to provide a direct, fast, and reliable method for such determinations. In this regard, some non-polar capillary GC columns were evaluated in an effort to find the appropriate stationary phase providing the optimum separation. The 100% dimethyl polysiloxane (Rtx-1) column gave better peak shapes and resolution of the two analytes within shorter analysis time compared to other non-polar columns, such as the 5% diphenyl–95% dimethyl polysiloxane (Rtx-5) and the 50% phenyl–50% methyl polysiloxane (Rxi-50) columns. Also, several temperature programs were evaluated, and a program showing the best resolution in a reasonable analysis time was selected. Programs with higher initial temperatures or higher ramp rates led to poor resolution whereas lower temperature ramps resulted in longer retention times and excessive peak tailing. PR eluted first (t_R = 10.19 ± 0.077 min) followed by TR (t_R = 12.27 ± 0.022 min), and the resolution (R_s) was found to be 7.12. An example chromatogram is shown in Figure 3.

Mass spectrum of PR (Figure 4A) is characterized by a base peak at *m/z* 109 formed by hydrogen transfer from the methyl group of the acetyl moiety to the ionized nitrogen followed by alpha cleavage. Subsequent rearrangement followed by the loss of the formaldehyde radical results in the formation of the more stable conjugated cyclopentadienylidene ammonium cation at *m/z* 80. Ionization of the amide oxygen followed by alpha cleavage gave the CH_3CO cation at *m/z* 43. Structures of the PR fragment ions are shown in Figure 5. Further proof of the suggested pathways was possible after the preparation of d_3-PR from condensation of 4-aminophenol and d_6-acetic anhydride. The mass spectrum in Figure 6 shows the analogous peaks at *m/z* 110, 81, and 46, respectively.

The mass spectrum of TR (Figure 4B) is characterized by ions at *m/z* 58 (base peak), *m/z* 188, and other ions of low relative abundance. Typical alpha cleavage between the tertiary amine and the cyclohexanol ring results in the imine ion at *m/z* 58 (27). Ionization of the π-electron in the benzene ring followed by rearrangements by the loss of both the hydroxyl group and the tertiary amine radicals results in the formation of the methoxy phenyl cyclohexene at *m/z* 188. Structures of the fragment ions of TR are shown in Figure 7.

Table I. Analytical Parameters for the Determination of PR and TR Using the Proposed Chromatographic Methods

Parameter	HPLC		GC–MS	
	PR	Tramadol	PR	Tramadol
Concentration range (µg/mL)	10–400	10–400	75–500	25–350
Intercept (a)	63.0×10^3	36.5×10^3	-17.4×10^4	-10.9×10^4
Sa*	98.1×10^3	41.2×10^3	16.6×10^4	22.4×10^4
Slope (b)	36132	24096	25189	50843
Sb†	454	191	415	900
RSD% of the slope	1.26	0.79	1.65	1.77
Correlation coefficient (r)	0.99960	0.99984	0.99851	0.99900
F‡	6314	15845	1677	2581
Significance F	59.8×10^{-8}	60.0×10^{-9}	16.4×10^{-6}	55.9×10^{-7}
LOD§ (µg/mL)	0.64	1.36	20.00	6.00
LOQ** (µg/mL)	2.13	4.53	66.70	20.00

* Standard deviation of the intercept. † Standard deviation of the slope.
‡ F equals the mean of squares due to regression divided by the mean of squares about regression (due to residuals). § Limit of detection. ** Limit of quantification.

Table II. Precision and Accuracy for the Determination of PR and TR Using the Proposed Chromatographic Methods

Analyte	Nominal value (µg/mL)	Found ± SD* (µg/mL)	RSD(%)†	Er(%)‡
HPLC:				
PR	50	50.78 ± 0.70	1.38	1.56
	100	99.62 ± 1.12	1.12	–0.38
	200	201.46 ± 1.78	0.88	0.73
Tramadol	50	50.05 ± 0.60	1.20	0.10
	100	100.12 ± 1.59	1.59	0.12
	200	199.58 ± 2.30	1.15	–0.21
GC–MS:				
PR	100	101.25 ± 1.54	1.52	1.25
	200	196.38 ± 3.12	1.59	–1.81
	300	297.57 ± 4.38	1.47	–0.81
Tramadol	100	99.56 ± 1.56	1.57	–0.44
	200	197.02 ± 2.58	1.31	–1.49
	300	294.75 ± 4.38	1.49	–1.75

* Mean ± standard deviation for five determinations.
† % Relative standard deviation. ‡ % Relative error.

Analytical performance of the proposed methods

Concentration ranges and calibration graphs

For both methods, the linearity of the detector response for the determination of PR and TR was evaluated by analyzing a series of different concentrations of each compound. Seven concentrations were chosen with triplicate injections for each concentration; this approach provided information on the variation in peak areas between samples of the same concentration. The linear regression equations were generated by least squares treatment of the calibration data. Table I presents the performance data and statistical parameters for the proposed methods including linear regression equations, concentration ranges, correlation coefficients, standard deviations of the intercept (S_a), and the slope (S_b). The analysis of variance (ANOVA) test for the regression lines reveals that for equal degrees of freedom, an increase in the variance ratio (F-values) means an increase in the mean of squares due to regression and decrease in the mean of squares due to residuals. The greater the mean of squares due to regression, the steeper the regression line. The smaller the mean of squares due to residuals, the less the scatter of experimental points around the regression line. Consequently, regression lines with high F values (low significance F) are much better than those with lower ones. Good regression lines show high values for both (r) and (F) statistical parameters (28).

Detection and quantification limits

According to the U.S. Pharmacopeia recommendations (4), limit of detection is defined as the concentration that has a signal-to-noise ratio of 3:1 whereas for limit of quantification the ratio considered is 10:1. These values were calculated and presented in Table I.

Precision and accuracy

The precision and accuracy for each method were examined at three concentration levels for the analyte by five replicate determinations for each concentration. The percentage relative standard deviation (RSD %) and the percentage relative error (E_r %) did not exceed 2%, proving the high repeatability and accuracy of the developed methods (Table II).

Selectivity

The selectivity of the proposed methods was tested by preparing different mixtures of PR and TR within the concentration ranges mentioned in Table I. These mixtures were of different ratios both above and below the normal ratio expected in the tablets. The laboratory-prepared mixtures were analyzed according to the previously mentioned HPLC and GC procedures. The recovery values, RSD %, and E_r % shown in Tables III and IV were satisfactory, thus validating the selectivity, precision, and accuracy of the developed methods.

Robustness

Robustness was examined by evaluating the influence of small variations in different experimental conditions such as working wavelength (± 2 nm), mobile phase pH (± 0.2 pH units), and organic strength (± 5%) for the HPLC method and temperature program ramp (± 2°C) for the GC–MS method. These variations did not have significant effect on the measured responses or the chromatographic resolution.

Stability

The stability of the PR and TR methanolic working solutions (for GC) was tested, and they were found to be stable for at least four days at room temperature. Also, the stability of the working solutions in the mobile phase (for HPLC) was verified, and no chromatographic changes were observed within 24 h at room temperature.

Table III. Determination of PR and TR Laboratory-made Mixtures Using the Proposed HPLC Method

Nominal value (µg/mL)		Found ± SD* (µg/mL)		RSD(%)†		Er(%)‡	
PR	TR	PR	TR	PR	TR	PR	TR
200	20	197.52 ± 1.98	20.55 ± 0.23	1.00	1.12	–1.24	2.75
200	25	198.40 ± 2.46	24.73 ± 0.27	1.24	1.09	–0.80	–1.08
200	40	198.54 ± 2.00	40.88 ± 0.55	1.01	1.35	–0.73	2.20
150	50	151.79 ± 1.91	49.81 ± 0.51	1.26	1.02	1.19	–0.38
100	50	98.42 ± 1.17	50.16 ± 0.74	1.19	1.48	–1.58	0.32
100	100	100.59 ± 1.55	101.29 ± 0.92	1.54	0.91	0.59	1.29
50	100	48.94 ± 0.50	100.98 ± 1.22	1.02	1.21	–2.12	0.98
50	150	48.33 ± 0.60	152.24 ± 1.44	1.24	0.95	–3.34	1.49

* Mean ± SD for 5 determinations. † % Relative standard deviation. ‡ % Relative error.

Table IV. Determination of PR and TR Laboratory-made Mixtures Using the Proposed GC–MS Method

Nominal value (µg/mL)		Found ± SD* (µg/mL)		RSD(%)†		Er(%)‡	
PR	TR	PR	TR	PR	TR	PR	TR
400	40	391.32 ± 3.52	40.61 ± 0.76	0.90	1.87	–2.17	1.53
400	50	403.04 ± 4.60	51.08 ± 1.09	1.14	2.13	0.76	2.16
250	50	255.10 ± 3.18	50.18 ± 0.90	1.25	1.79	2.04	0.36
300	100	292.41 ± 2.91	101.66 ± 0.97	1.00	0.95	–2.53	1.66
200	100	196.12 ± 3.12	97.89 ± 1.12	1.59	1.14	–1.94	–2.11
200	200	199.04 ± 3.54	201.06 ± 3.28	1.78	1.63	–0.48	0.53
100	200	102.00 ± 1.87	195.60 ± 1.94	1.83	0.99	2.00	–2.20
100	300	101.12 ± 1.68	304.65 ± 3.90	1.66	1.28	1.12	1.55

* Mean ± SD for 5 determinations. † % Relative standard deviation. ‡ % Relative error.

Analysis of pharmaceutical preparation

The developed chromatographic methods were applied for the assay of the two drugs in their combined pharmaceutical formulation (Tramol-Plus tablets). Table V shows the results obtained for the proposed methods as well as the reference derivative spectrophotometric method (21). The assay results showed good precision and accuracy as indicated from % recovery, SD, and RSD (%) values. No interfering peaks were observed in the HPLC or GC chromatograms of the tablets. Results obtained by the developed methods were statistically compared with those of the previously

Table V. Application of the Proposed Chromatographic Methods in the Analysis of PR and TR Commercial Tablets

PR	HPLC	GC–MS	Reference method
% Recovery ± SD*	96.27 ± 1.02	96.59 ± 1.21	95.15 ± 1.17
RSD(%)	1.06	1.25	1.23

ANOVA

Source of Variation	Sum of Squares	Degrees of Freedom	Mean of Squares	F†
Between Groups	5.682	2	2.841	2.198
Within Groups	15.510	12	1.293	
Total Variation	21.193	14		

TR	HPLC	GC–MS	Reference method
% Recovery ± SD*	95.73 ± 0.95	95.37 ± 1.09	94.84 ± 1.01
RSD(%)	0.99	1.14	1.07

ANOVA

Source of Variation	Sum of Squares	Degrees of Freedom	Mean of Squares	F†
Between Groups	1.998	2	0.999	0.965
Within Groups	12.428	12	1.036	
Total	14.427	14		

* Mean ± standard deviation for five determinations.
‡ The theoretical value for F equals 3.885 at P = 0.05.

published derivative spectrophotometric method using single factor analysis of variance (ANOVA) test (29), which is considered a useful statistical tool for comparison of recovery data obtained from more than two methods. The calculated F-values did not exceed the critical value for either of the two drugs, indicating no significant differences between the proposed methods together with the reference method.

Conclusion

In this study, two simple, rapid, and selective chromatographic procedures were established for the simultaneous determination of PR and TR in laboratory-prepared mixtures as well as in commercially available tablets. The HPLC method made the use of an embedded amide reverse C_8 stationary-phase with a low organic modifier content (10% acetonitrile) mobile phase. Additionally, the liquid chromatographic method reported here showed wider linearity ranges and better sensitivity compared to the previously published HPLC methods. The GC–MS method is direct, requiring minimal sample preparation and made use of the total ion current detection. Both methods need no derivatization or pretreatment of the target compounds. The described validated chromatographic methods offer selectivity advantage over the spectrophotometric-based non-separation methods. Finally, the proposed methods were found accurate and precise, thus making them convenient for quality control purposes.

References

1. B. Hinz and K. Brune. Antipyretic Analgesics: Nonsteroidal Antiinflammatory Drugs, Selective COX-2 Inhibitors, Paracetamol and Pyrazolinones. *Handbook of Experimental Pharmacology.* **177(Analgesia):** 65–93 (2006).
2. A. Bertolini, A. Ferrari, A. Ottani, S. Guerzoni, R. Tacchi, and S. Leone. Paracetamol: New Vistas of an Old Drug. *CNS Drug Reviews* **12(3–4):** 250–275 (2006).
3. S. Grond and A. Sablotzki. Clinical Pharmacology of Tramadol. *Clin. Pharmacokinet.* **43(13):** 879–923 (2004).
4. The United States Pharmacopeia 30th edition, The National Formulary, 25th edition "The Official Compendia of Standards", United States Pharmacopeial Convention, Inc., Asian Edition, Washington, D.C. (2007).
5. The British Pharmacopoeia, Volumes II and III, Her Majesty's Stationery Office, London, UK (2007).
6. M. Espinosa Bosch, A.J. Ruiz Sánchez, F. Sánchez Rojas, and C. Bosch Ojeda. Determination of paracetamol: Historical evolution. *J. Pharm. Biomed. Anal.* **42:** 291–321 (2006).
7. W.F. Kartinasari, T. Palupi, and G. Indrayanto. HPLC determination and validation of tramadol hydrochloride in capsules. *J. Liq. Chromatogr. Relat. Technol.* **27(4):** 737–744 (2004).
8. M.R. Rouini, Y.H. Ardakani, F. Soltani, H.Y. Aboul-Enein, and A. Foroumadi. Development and validation of a rapid HPLC method for simultaneous determination of tramadol, and its two main metabolites in human plasma. *J. Chromatogr. B* **830(2):** 207–211 (2006).
9. K.A. Hadidi, J.K. Almasad, T. Al-Nsour, and S. Abu-Ragheib. Determination of tramadol in hair using solid phase extraction and GC–MS. *Forensic Sci. Int.* **135:** 129–136 (2003).
10. Y.F. Sha, S. Shen, and G.L. Duan. Rapid determination of tramadol in human plasma by headspace solid-phase microextraction and capillary gas chromatography–mass spectrometry. *J. Pharm. Biomed. Anal.* **37:** 143–147 (2005).
11. J. Krzek and M. Starek. Quality assessment for tramadol in pharmaceutical preparations with thin layer chromatography and densitometry. *Biomed. Chromatogr.* **18(8):** 589–599 (2004).
12. U.B. Soetebeer, M.O. Schierenberg, H. Schulz, G. Gruenefeld, P. Andresen, and G. Blaschke. Assay of tramadol in urine by capillary electrophoresis using laser-induced native fluorescence detection. *J. Chromatogr. B* **745(2):** 271–278 (2000).
13. J.G. Li and H.X. Ju. Simultaneous determination of ethamsylate, tramadol and lidocaine in human urine by capillary electrophoresis with electrochemiluminescence detection. *Electrophoresis* **27(17):** 3467–3474 (2006).
14. P. Norouzi, R. Dinarvand, M.R. Ganjali, and A.S.E. Meibodi. Application of Adsorptive Stripping Voltammetry for the Nano-Level Detection of Tramadol in Biological Fluids and Tablets Using Fast Fourier Transform Continuous Cyclic Voltammetry at an Au Microelectrode in a Flowing System. *Anal. Lett.* **40(11):** 2252–2270 (2007).
15. E.M.P.J. Garrido, J.M.P.J. Garrido, F. Borges, and C. Delerue-Matos. Development of electrochemical methods for determination of tramadol–analytical application to pharmaceutical dosage forms. *J. Pharm. Biomed. Anal* **32(4–5):** 975–981 (2003).
16. H. Hopkala, G. Misztal, and A. Wieczorek. Tramadol selective PVC membrane electrodes and their analytical application. *Die Pharmazie* **53(12):** 869–871 (1998).
17. H.E. Abdellatef, M.M. El-Henawee, H.M. El-Sayed, and M.M. Ayad. Spectrophotometric and spectrofluorimetric methods for analysis of tramadol, acebutolol and dothiepin in pharmaceutical preparations. *Spectrochim. Acta A* **65(5):** 1087–1092 (2006).
18. H.E. Abdellatef. Kinetic spectrophotometric determination of tramadol hydrochloride in pharmaceutical formulation. *J. Pharm. Biomed. Anal.* **29(5):** 835–842 (2002).
19. T. Zhu, L. Ding, X. Guo, L. Yang, and A. Wen. Simultaneous Determination of Tramadol and Acetaminophen in Human Plasma by LC–ESI–MS. *Chromatographia* **66:** 171–178 (2007).
20. Z. Tan, D. Ouyang, G. Zhou, L. Wang, Z. Li, D. Wang, G. Chen, S. Huang, Y. Liu, D. Hu, and H. Zhou. Determination of tramadol and paracetamol in human plasma by liquid chromatography ion trap mass spectrum. *Yaowu Fenxi Zazhi* **25(7):** 795–798 (2005).
21. K.K. Srinivasan, J. Alex, A.A. Shirwaikar, S. Jacob, M.R. Sunil Kumar, and S.L. Prabu. Simultaneous derivative spectrophotometric estimation of aceclofenac and tramadol with paracetamol in combination solid dosage forms. *Indian J. Pharm. Sci.* **69(4):** 540–545 (2007).
22. Y. Li, X. Wan, G. Wang, and J. Cui. Simultaneous determination of tramadol hydrochloride and paracetamol in their compound sustained release tablets by principal component regression spectrophotometry. *Zhongguo Yaoxue Zazhi (Beijing, China)* **41(20):** 1594–1595 (2006).
23. X. Liu, J. Shi, Y. Liu, and Z. He. Simultaneous determination of contents of tramadol hydrochloride and paracetamol in their compound tablets by HPLC. *Shenyang Yaoke Daxue Xuebao* **21(2):** 111–113 (2004).
24. L. Hong and J. Li. Determination of compound tramadol hydrochloride tablets and its related substances by HPLC. *Zhongguo Yiyao Gongye Zazhi* **36(5):** 293–95 (2005).
25. D. Peng, K. Huang, Y. Liu, and M. Li. Simultaneous determination of two constituents in compound tramadol tablets by HPLC. *Zhongguo Xinyao Zazhi* **15(2):** 120–122 (2006).
26. D.J. Speed, S.J. Dickson, E.R. Cairns, and N.D. Kim. Analysis of paracetamol using solid-phase extraction, deuterated internal standards, and gas chromatography-mass spectrometry. *J. Anal. Toxicol.* **25(3):** 198–202 (2001).
27. F.W. McLafferty, and F. Turecek. Interpretation of Mass Spectra, Fourth edition University Science Books, Sausalito, CA, (1993).
28. P. Armitage, and G. Berry. *Statistical Methods in Medical Research*, Third Edition, Blackwell, Oxford, pp. 283–285 (1994).
29. J.N. Miller, and J.C. Miller. *Statistics and Chemometrics for Analytical Chemistry,* Fourth Edition, p. 57–64,77,78 (2000).

Manuscript received May 1, 2008;
Revision received June 24, 2008.