ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
20 (1999) 705–716

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Development and validation of an HPLC assay for fentanyl and related substances in fentanyl citrate injection, USP

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Received 16 November 1998; received in revised form 23 February 1999; accepted 5 March 1999

Abstract

The stability indicating properties of the USP method for the assay of fentanyl in fentanyl citrate injection were evaluated [1] by analyzing fentanyl drug substance and product after acid, hydrogen peroxide, heat, and light treatment. *N*-phenyl-*N*-(4-piperidinyl)propionamide (PPA), which is a known degradation product/process impurity of fentanyl, was not adequately resolved from the fentanyl peak, and mobile phase adjustments did not improve the resolution (Fig. 1). Therefore, the USP method did not meet the requirements for a stability-indicating assay. In addition, the wavelength in the USP method was too high (230 nm) to provide adequate levels for the quantitation of the related substances of fentanyl and, in addition, the acetate ions in the mobile phase could interfere with a lower wavelength detection. An isocratic, reversed phase, stability indicating, high performance liquid chromatographic (HPLC) method for the assay of fentanyl and related substances in fentanyl citrate injection, USP has been developed and validated. The chromatographic conditions employed an Inertsil C8, 5 column (25 cm × 4.6 mm), a mobile phase of aqueous perchloric acid [0.23%, w/v]-acetonitrile [65:35, v/v], and ultraviolet (UV) detection at 206 nm. Under the chromatographic conditions of the method, PPA and seven other known process impurities were separated from the active. Degradation studies showed that the active eluted as a spectrally pure peak resolved from its degradation products.

Keywords: LC; Assay; Fentanyl; Related substances; Injection

1. Introduction

Fentanyl [*N*-phenyl-*N*-(1-(2-phenylethyl)piperidyl)propanamide] is a potent synthetic opioid analgesic that is ≈100 times more potent than

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0731-7085/99/$ - see front matter
PII: S0731-7085(99)00077-1

morphine [2]. Its main therapeutic applications are intravenous or intramuscalar analgesia and sedation, and has been widely used for the purpose of neuroleptic analgesia and surgical anesthesia at doses ranging from 2 to 50 g ml^{-1}. Fentanyl citrate injection, USP is formulated with 50 g ml^{-1} of fentanyl base (as the citrate salt) in water and may also contain sodium hydroxide and/or hydrochloric acid for pH adjustment to 4.7 [3–5]. The pH range specified by the USP is from 4.0 to 7.5.

This work was conducted in order to develop and validate a stability-indicating HPLC assay method that allows for the resolution, detection, and quantitation of known related substances of fentanyl.

2. Experimental

2.1. Materials

HPLC grade acetonitrile from Mallinckrodt was used to prepare the mobile phase. Perchloric acid of reagent grade quality from Mallinckrodt and in-house Milli-Q water were used to prepare the aqueous component of the mobile phase. Fentanyl citrate standard and authentic samples of all related substances were obtained from Johnson-Matthey. The related substance 2-bromoethylbenzene was obtained from Aldrich Chemical Co. The analytical sample (fentanyl citrate injection, USP, 0.050 mg ml^{-1}) was supplied by the AAI Formulations Development Laboratory. Structures and names of these compounds are given in Fig. 2.

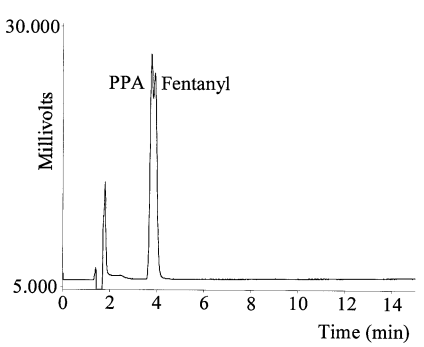

Fig. 1. Chromatogram of a fentanyl citrate sample solution after acid degradation (USP method).

2.2. Equipment

An HPLC system consisting of a Hitachi Model L6200a Intelligent pump plus an Alcott Model 728 autosampler, ACCESS*CHROM 1.9 chromatography data software with PE Nelson A/D interface system, and an Applied Biosystems 759A variable wavelength UV detector was used. A Waters 991 photodiode array detector was also utilized for the degradation studies.

All separations were achieved using 25 cm × 4.6 mm ID, 5μ C8 Inertsil columns (obtained from Phenomenex or Alltech). All sample and standard solutions were chromatographed at ambient temperature using a mixture of aqueous perchloric acid (0.23%, w/v)-acetonitrile (65:35, v/v) as the mobile phase, with detection at 206 nm, a flow rate of 1.0 ml min^{-1}, and an injection volume of 60 μl. Peak area responses were used for the quantitation of the active and the related substances.

2.3. Development of the chromatographic separation

Other reported methods for the analysis of fentanyl citrate and its related substances were found to be lacking adequate sensitivity, and no complete validation of a stability indicating method has been reported to include the related substances of fentanyl, especially at low levels [3–5].

Initially, the stability-indicating properties of the USP method for the assay of fentanyl in fentanyl citrate injection [1] were evaluated by analyzing fentanyl citrate drug substance and drug product after acid, hydrogen peroxide, heat, and light treatments (Table 1). The degradation product from acidic treatment (as well as process impurity) 4-anilino-*N*-phenethyl-piperiding (PPA) was not adequately resolved from the active peak (Fig. 1). The results were confirmed by spiking a fentanyl standard solution with a known amount of the PPA impurity and analyzing the sample

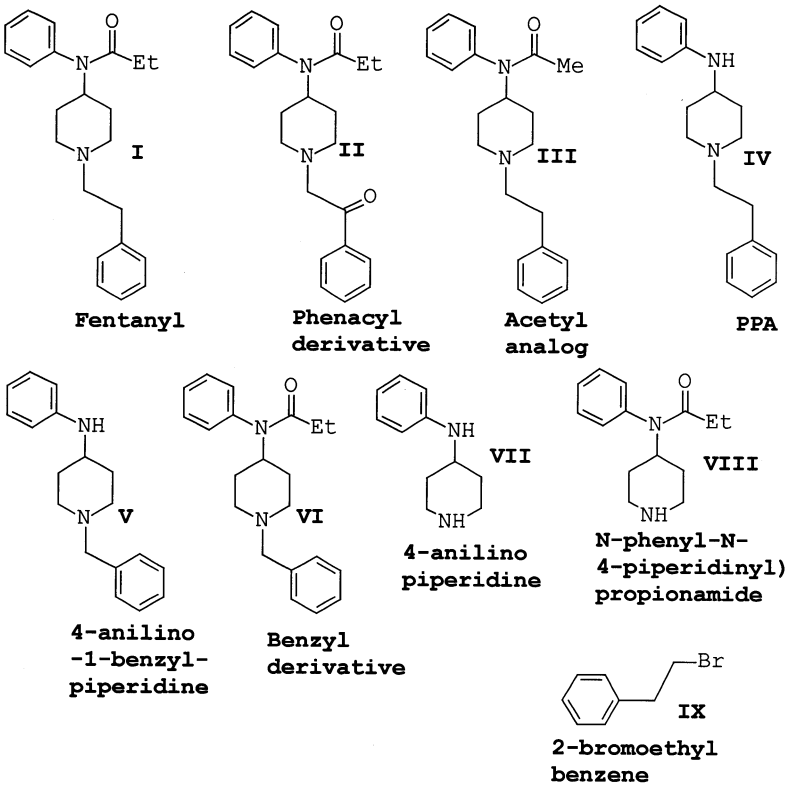

Fig. 2. Structures of fentanyl and related substances.

Table 1
Recovery of degraded fentanyl citrate drug substance and injection samples (USP method)[a]

Condition	Time	% Recovered	RRT of degradants
Acid (drug substance) 3 N HCl, 90°C	2 h	78.5	0.96[b]
Acid (injection) 3 N HCl, 90°C	2 h	118.0[c]	NR[c]
3% Hydrogen peroxide (drug substance) room temperature	45 min	100.0	ND
3% Hydrogen peroxide (injection) room temperature	45 min	100.5	ND
Heat, 90°C (drug substance)	6 h	99.7	ND
Heat, 90°C (injection)	6 h	100.8	ND
Light 1000 foot candles (drug substance)	24 h	101.0	ND
Light 1000 foot candles (injection)	24 h	99.9	ND

[a] ND, none detected.
[b] Peak was identified as PPA.
[c] PPA was not resolved from the active; purity check by diode-array detector.

using a diode-array detector. Changes to the chromatographic conditions did not efficiently resolve the two peaks. Therefore, The USP method did not meet the requirements for a stability-indicating assay and a new method needed to be developed and validated.

It was found that perchloric acid is an excellent ion-pairing agent that significantly improves the

peak shape of the active and, therefore, provides enhanced signal for quantitation at very low levels. Chromatographic retention of fentanyl, employing reversed phase columns, perchloric acid as the ion-pairing agent, and acetonitrile as the organic modifier resulted in efficient chromatography (tailing, theoretical plates, etc.). This mobile phase composition was transparent at low UV wavelengths (e.g. 206 nm), and, therefore, allowed for quantitation at the 0.1% level of all related substances.

2.3.1. Preparation of mobile phase

Perchloric acid (0%, w/w, 2.0 ml) was carefully added to 1.0 l water and mixed well. A portion of the resulting solution (650 ml) was mixed with acetonitrile (350 ml) and degassed with helium sparge.

2.3.2. Preparation of standard solutions

Approximately 39.3 mg of fentanyl citrate reference standard was dissolved in 100 ml of water. An aliquot (5.0 ml) of the stock standard solution was diluted in 25 ml of water to provide a concentration of about 0.050 mg ml^{-1} fentanyl free base.

2.3.3. Preparation of sensitivity solution

An aliquot (1.0 ml) of the working standard solution was diluted in 100 ml of water. The resulting solution was rediluted (1.0 ml to 50 ml of water) to provide a concentration of about 0.01 mg ml^{-1} fentanyl free base.

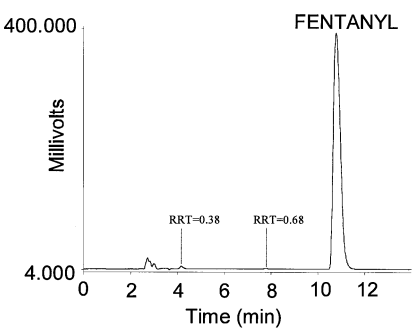

Fig. 3. Example chromatogram of a fentanyl citrate sample preparation.

2.3.4. Preparation of samples

No dilutions were performed for assay samples.

Accuracy/recovery samples were prepared by dissolving $\approx$19.7, 39.3, and 59.0 mg of fentanyl citrate reference standard in water and diluting the resulting solutions to obtain concentrations at levels corresponding to 50, 100, and 150% of the label claim, respectively. In addition, a solution of PPA was spiked into fentanyl citrate solutions (prepared at 0.050 mg fentanyl free base ml^{-1}) at levels corresponding to 0.1 to 3.0% of the fentanyl nominal concentration.

Degradation samples were prepared by subjecting concentrated fentanyl citrate aqueous solutions (5.0 ml of stock standard solution) to acid, base, hydrogen peroxide, heat, and UV light (254 nm) in 25 ml volumetric flasks. After the degradation treatments were completed, all samples were allowed to cool to room temperature, neutralized with acid/base (if needed) and prepared according to the assay procedures.

3. Results

3.1. System precision

The study was performed by making ten replicate injections of the standard solution and ten replicate injections of a sample preparation solution. The relative standard deviation of the area of the fentanyl peak was found to be 0.4 and 0.2%, respectively. An example chromatogram of fentanyl is shown in Fig. 3.

3.2. Intermediate precision

Intermediate precision for the drug product was determined by the assay of five sample preparations on two separate days, by two different analysts, on different chromatographic systems. Table 2a–b summarizes the chromatographic parameters obtained during the study, as well as the results for the two analysts for the fentanyl citrate injection, USP.

Table 2
Intermediate precision for fentanyl citrate injection[a]

Sample	Analyst #1 Day #1 System #1 % Label claim	Analyst #2 Day #2 System #2	Analyst #1 Day #1 System #1 % Area PPA[b]	Analyst #2 Day #2 System #2		
1	95.0	95.3	ND	ND		
2	95.1	95.6	ND	ND		
3	95.0	95.5	ND	ND		
4	95.0	95.2	ND	ND		
5	95.3	95.5	ND	ND		
Mean (5)	95.1	95.4	ND	ND		
% RSD	0.1	0.2	N/A	N/A		
Totals						
Mean (10)	95.3		ND			
% RSD	0.2		N/A			
	% Total impurities		% Individual impurities[c]			
1	0.82	0.81	0.50[d]	<0.10%[f]	0.43[e]	0.12[f]
2	0.83	0.84	0.53	<0.10%	0.45	0.12
3	0.82	0.86	0.53	<0.10%	0.46	0.12
4	0.75	0.84	0.53	<0.10%	0.46	0.13
5	0.76	0.82	0.53	<0.10%	0.44	0.13
Mean (5)	0.80	0.83	0.52	<0.1%	0.45	0.12
% RSD	4.8	2.7	2.6		2.9	4.4
Totals						
Mean (10)	0.81					
% RSD	4.3					

Summary of chromatographic parameters obtained during intermediate precision studies

	Tailing factor	Theoretical plates	% RSD[g]	RRT[h]
Analyst #1	1.44	6152	0.4	0.61
Analyst #2	1.56	7656	0.1	0.58

[a] Note: each value is the mean of five injections; ND, none detected; N/A, not applicable.
[b] PPA: *N*-phenyl-*N*-(4-piperidinyl)-propionamide.
[c] Individual impurities >0.1% were reported.
[d] Relative retention time (RRT), 0.39.
[e] Relative retention time (RRT), 0.37.
[f] Relative retention time (RRT), 0.68.
[g] %RSD of the peak area responses of five standard injections.
[h] Retention time of PPA relative to fentanyl.

3.3. Range of linearity (fentanyl/PPA)

The linearity parameters of the curve for the fentanyl peak area response versus the fentanyl concentration were studied in the concentration range corresponding to about 40 to 160% of the nominal analytical concentration of 0.050 mg ml^{-1}.

The linearity parameters of the area response of the fentanyl degradation/process impurity, PPA,

Table 3
Parameters of linearity of fentanyl

% Nominal analytical concentration	Concentration (mg ml^{-1})	Peak area	Calculated peak area	Residual[a]	Response factor[b]
40	0.020	3350020	3361970	−11950	1666×10^5
		3358270	3361970	−3699	1670×10^5
80	0.040	6725010	6704780	20220	1672×10^5
		6707760	6704780	2979	1668×10^5
100	0.050	8375490	8376190	−701.6	1666×10^5
		8365780	8376190	−10420	1664×10^5
120	0.060	10080500	10047600	32880	1671×10^5
		10032000	10047600	−15650	1663×10^5
160	0.080	13420100	13390400	29670	1668×10^5
		13347100	13390400	−43330	1659×10^5
Y-intercept, 19156.8					
Slope, 166230 000					
Correlation coefficient, 0.999979					
% *Y*-intercept[c], 0.2					

[a] Residual, Peak area − calculated peak area.
[b] Response factor, peak area/concentration.
[c] % *Y*-intercept, (*Y*-intercept, peak area at 100% nominal anal. conc.) × 100.

Table 4
Parameters of linearity of PPA

% Nominal analytical concentration	Concentration (μg ml^{-1})	Peak area	Calculated peak area	Residual[a]	Response factor[b]
0.1	0.048	5772	8126.13	−2354	120300
		6396	8126.13	−1730	133300
0.5	0.24	30724	32677.0	−1953	128000
		30938	32677.0	−1739	128900
1.0	0.48	70232	63365.6	6866	146300
		70840	63365.6	7474	147600
2.0	0.96	121440	124743	−3303	126500
		120554	124743	−4189	125600
3.0	1.50	194395	193792	602.9	129600
		194116	193792	323.9	129400
Y-intercept, 1988.41					
Slope, 127877					
Correlation coefficient, 0.998369					
% *Y*-intercept[c], 2.8					

[a] Residual, peak area − calculated peak area.
[b] Response factor, peak area/concentration.
[c] % *Y*-intercept, (*Y*-intercept/peak area at 100% nominal anal. conc.) × 100.

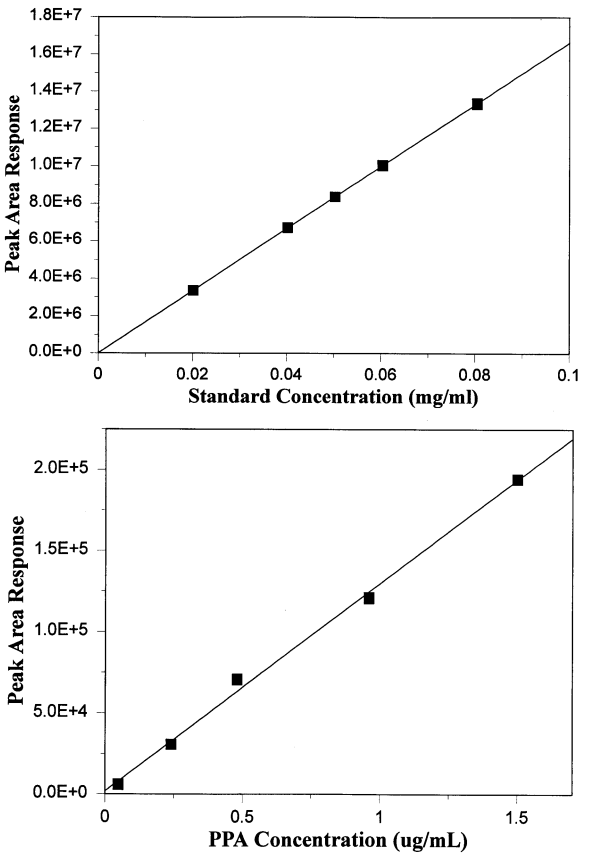

Fig. 4. Linearities of fentanyl and PPA.

Table 5
Relative response factor of PPA

	Peak area responses	
	Fentanyl	PPA[a]
	8456958	73016
	8445507	71833
	8494372	71802
	8460604	73766
	8474738	71694
Mean (5)	8466436	72422
% RSD	0.2	1.3

[a] Relative response factor (Rrf) = 0.90.

were studied from ≈ 0.1 to 3.0% of the nominal fentanyl concentration of 0.050 mg ml^{-1} (Tables 3 and 4, Fig. 4).

3.4. Limits of detection (LOD) and quantitation (LOQ)

The limits of detection and quantitation were determined by serial dilutions of fentanyl citrate and PPA stock solutions in order to obtain signal–noise ratios of $\approx 10{:}1$ for LOQ and 3:1 for LOD. The LOD and LOQ values of fentanyl were found to ≈ 0.010 mg ml^{-1} (signal/noise = 3.3) and 0.040 mg ml^{-1} (signal/noise = 11.0), respectively. The LOD and LOQ values of PPA were found to be ≈ 0.014 mg ml^{-1} (signal/noise = 3.0) and 0.048 mg ml^{-1} (signal/noise = 10.1), respectively.

3.5. Relative response factor for PPA

The response factor peak area concentration of PPA was compared to the response factor of fentanyl. PPA was injected at a concentration of 0.5 g ml^{-1} (1% of the fentanyl nominal concentration), while fentanyl was injected at a concentration of 50 mg ml^{-1} (nominal concentration). The average of five replicate injections for PPA and five replicate injections for fentanyl were used in the calculation. The concentration of PPA was corrected for the chromatographic purity of the PPA standard, which was assigned as 98.4% based on the area percent calculation of injections of PPA solutions with concentrations of 50 mg ml^{-1}. The relative response factor of PPA to fentanyl was calculated as 0.90 (Table 5).

3.6. Accuracy/recovery studies

Triplicate solutions of fentanyl were prepared at each recovery level and chromatographed versus a fentanyl standard solution. In addition, the recoveries of the PPA-spiked fentanyl citrate solutions were calculated versus the area of the fentanyl contained within the samples and adjusted by the relative response factor of PPA. The recovery results for fentanyl and PPA are summarized in Table 6. The somewhat lower recoveries of PPA are probably due to the calculation of the response factor (see previous section). Percentage peak area responses were used in the determination of its purity, which could introduce some error in the calculation of the response factor (0.90).

3.7. Stability of analytical solutions

The stability of standard solutions was monitored by analyzing standard solutions aged at room temperature, while protected from light, against freshly prepared standards. The results demonstrated that fentanyl citrate in working standard solutions was stable for at least ten days. The stock standard solution was found to be stable for at least 29 days.

Stability of the sample was evaluated by assaying a sample solution spiked with PPA immediately after its preparation and again, against a fresh standard, after it had aged at room temperature while protected from light. These results indicated that sample solutions were stable for at least 5 days (Table 7). During the stability studies no additional/growing peaks were developed and no changes in the chromatography were observed.

3.8. Selectivity

No interference was observed in the region of the fentanyl or PPA peaks in injections of diluent and all related substances were resolved from each other and from the fentanyl peak (Figs. 5 and 6). It was observed that one related substance eluted in the solvent front and another impurity was retained on the column (compounds VII and IX, respectively).

Table 6
Accuracy/recovery for fentanyl citrate and PPA from fentanyl samples

Sample	Spiking level	% Recovery (fentanyl)	Mean (3)	% RSD
1	50	100.5		
2	50	100.5	100.6	0.2
3	50	100.8		
4	100	99.7		
5	100	99.4	99.6	0.2
6	100	99.7		
7	150	98.8		
8	150	99.9	99.5	0.6
9	150	99.9		
	Mean (9)	99.9		
	% RSD	0.6		
Sample	Approximate level %	% Recovery (PPA)	Mean (3)	% RSD
10	0.1	99.4		
11	0.1	102.5	97.2	6.9
12	0.1	89.7		
13	0.5	94.9		
14	0.5	92.4	92.0	3.3
15	0.5	88.8		
16	1.0	90.8		
17	1.0	90.7	90.6	0.3
18	1.0	90.3		
19	3.0	91.1		
20	3.0	91.4	91.1	0.4
21	3.0	90.7		
	Mean (12)	92.7		
	% RSD	4.5		

Table 7
Stability of fentanyl citrate in analytical solutions

Working standard solution					
Standard, % recovered					
Initial	1 Day	2 Days	7 Days	8 Days	10 Days
100.0	99.0	100.5	100.2	100.2	101.7
Stock standard solution					
Standard, % recovered					
Initial	8 Days	15 Days	19 Days	29 Days	
100.0	100.5	101.4	99.2	100.7	
Sample solution					
Component:	Initial	5 Days			
Fentanyl	100.0	99.3			
PPA	0.46	0.45			

3.9. Robustness

The effect of variations in the concentrations of perchloric acid and acetonitrile of the mobile phase on the reproducibility, tailing factor, theoretical plates for the fentanyl peak and the resolution between fentanyl and PPA was studied. The data of Table 8 demonstrate that within the studied mobile phase variations the chromatographic parameters of the method do not affect the column efficiency and provide acceptable tailing for the fentanyl peak and sufficient resolution between the active and PPA.

3.10. Degradation studies

Forced degradation studies were performed to provide an indication of the stability-indicating properties and specificity of the method. Intentional degradation was attempted using acid, base, hydrogen peroxide, heat, and UV light (254 nm). After the degradation treatments were completed, all samples were allowed to cool to room temperature, neutralized with acid/base (if needed) and prepared according to the assay procedures.

The degraded solutions were analyzed against freshly prepared standards following the chromatographic conditions. The percent of active recovered as well as the relative retention times for all degradation products are shown in Table 9. Degradation peaks, where observed, were resolved from the fentanyl peak (Fig. 7). Diode-array spectra of the fentanyl peak, taken during the upslope, apex, and downslope did not reveal any coeluting degradation products or impurities. A representative diode-array spectrum of a sample preparation is shown in Fig. 8.

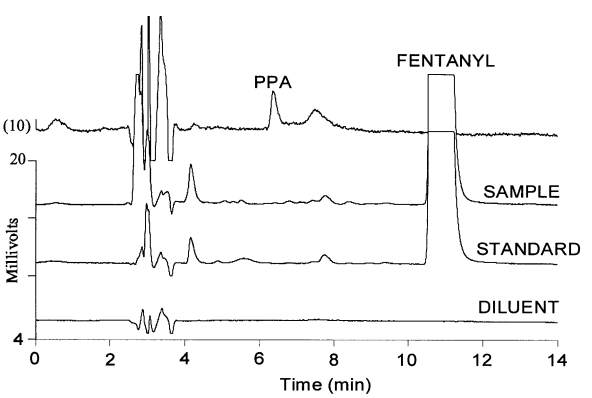

Fig. 5. Overlay of chromatograms of diluent, standard solution, sample solution, and a PPA sample (0.1%).

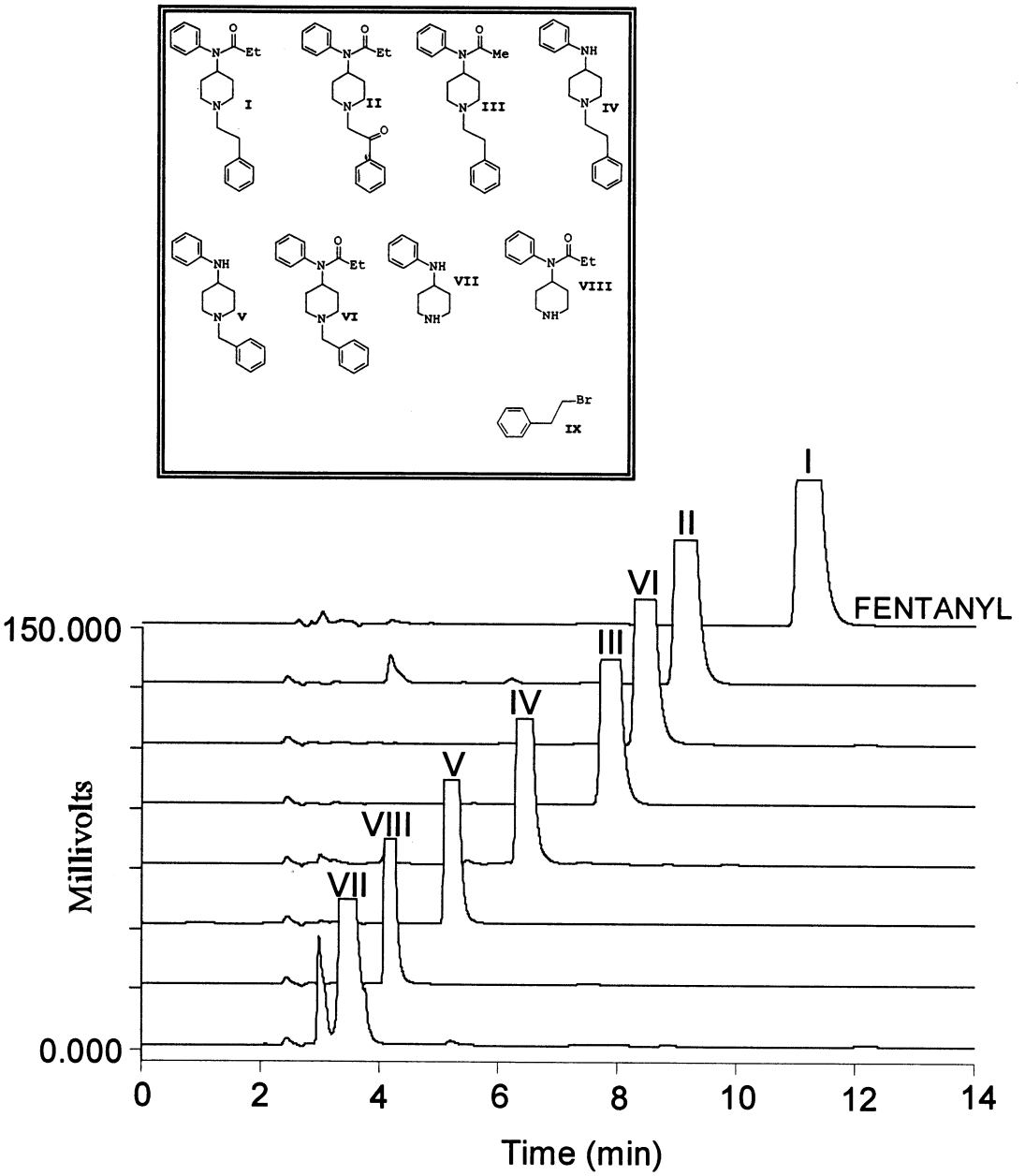

Fig. 6. Overlay of chromatograms of fentanyl and related substances.

4. Conclusions

The correlation coefficient and % y-intercept of the linearity curve of a fentanyl standard were found to be 0.999979 and 0.2, respectively. The linearity curve for PPA demonstrated a correlation coefficient of 0.998369 with a 2.8% *Y*-intercept. The limits of detection (LOD) and quan-

Table 8
Chromatographic parameters for fentanyl under varied conditions[a]

Condition #	% RSD[b]	Theoretical plates	Tailing factor	Resolution[c]
1	0.2	7492	1.59	15.9
2	0.1	7421	1.62	11.8
3	0.1	7564	1.67	8.0
4	0.2	7356	1.70	10.9

Condition #	
1	Aqueous perchloric acid [0.23%, w/v]:Acetonitrile [70:30, v/v]
2	Aqueous perchloric acid [0.25%, w/v]:acetonitrile [65:35, v/v]
3	Aqueous perchloric acid [0.23%, w/v]:acetonitrile [60:40, v/v]
4	Aqueous perchloric acid [0.21%, w/v]:acetonitrile [65:35, v/v]

[a] Note: each value represents the mean of three injections.
[b] % RSD of fentanyl peak area response.
[c] Resolution between fentanyl and PPA.

Table 9
Percent recovery of degraded fentanyl citrate samples

Condition	Time	% Recovered	RRT of degradants
Acid 0.5 N HCl, 80°C	1 day	95.7	0.59[a]
Base 0.5 N NaOH, 80°C and room temperature	5 days 14 days	97.3	0.39, 0.40, 0.59[a], 0.79
3% Hydrogen peroxide 80°C	3 h	69.6	0.38, 0.40, 0.44, 0.45, 0.49, 0.52, 0.55[a], 0.63, 0.66, 0.69, 0.78, 1.13, 1.20, 1.35
Heat 80°C and room temperature	5 days 14 days	98.3	0.63, 0.78
Light 1000 foot candles and dark	13 days 6 days	69.4	0.39, 0.41, 0.47, 0.50, 0.53, 0.56[a], 0.59, 0.67, 0.70, 0.79

[a] Peak was identified as PPA

titation (LOQ) for the active were 0.010 μg ml^{-1} and 0.040 μg ml^{-1}, respectively. The corresponding values for PPA were calculated as 0.014 μg ml^{-1} (LOD) and 0.048 μg ml^{-1} (LOQ). Accuracy/recovery, measured by triplicate preparations at the 50, 100, and 150% levels, showed a mean of 99.9% with a relative standard deviation (RSD) of 0.6%. Method precision/ruggedness for ten assays with two different analysts and systems was 95.3% (0.2% RSD) for potency and 0.81% (4.3% RSD) for total impurities.

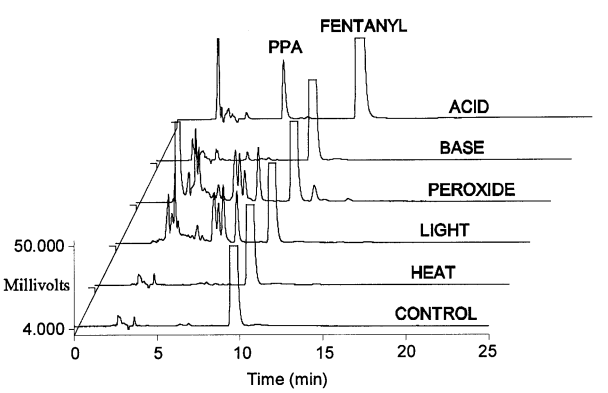

Fig. 7. Overlay of chromatograms from fentanyl degradation study.

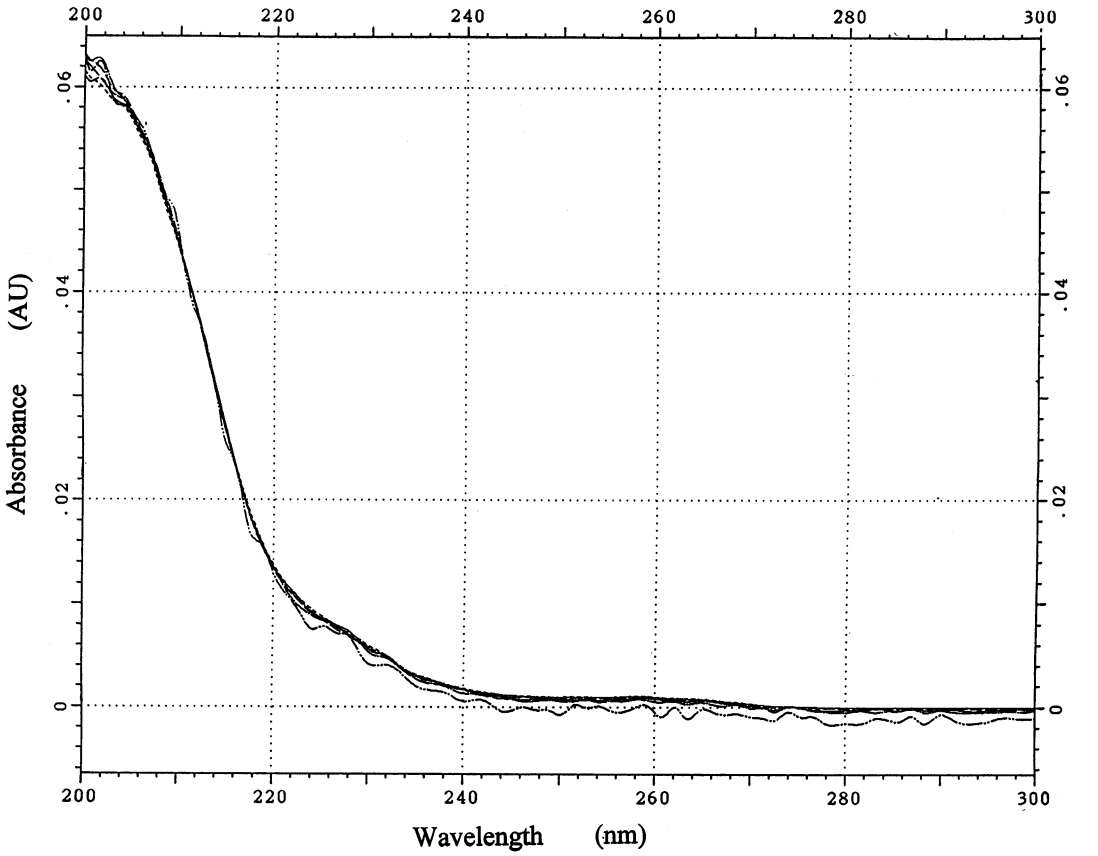

Fig. 8. Example diode-array spectrum/base degradation.

The stock standard solution (0.25 mg ml^{-1} fentanyl free base) and working standard solution (50 μg ml^{-1}) were found to be stable for 29 and 10 days, respectively, at room temperature and protected from light. A fentanyl citrate sample solution spiked with 0.5% PPA showed a five day stability for both the active and the PPA degradation product at both room temperature and in the dark.

Acknowledgements

The authors wish to acknowledge Dr Tanya Toney-Parker and the rest of the staff at the AAI Formulations Development Laboratory (FDL) for their support.

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