

Comparison of Three Analytical Methods for Quality Control of Clozapine Tablets

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Three different analytical methods for the quality control of clozapine in commercial formulations were developed and compared: a liquid chromatographic (LC) method with UV detection, a capillary zone electrophoretic (CZE) method, and a linear scan voltammetric (LSV) method. The isocratic LC procedure used a C₁₈ reversed-phase column; the CZE method used an uncoated fused-silica capillary and phosphate buffer containing polyvinylpyrrolidone as the background electrolyte; the LSV method analyzed clozapine solutions with acidic phosphate buffer as the supporting electrolyte. The 3 methods gave similar and satisfactory results, in terms of precision and accuracy. Repeatability and intermediate precision were good (RSD% < 2.2) and accuracy, resulting from recovery studies, was between 98 and 102%. The rapidity of analysis was high for all 3 methods, especially for the LSV.

Clozapine (8-chloro-11-(4-methyl-piperazin-1-yl)-5H-dibenzo[b,e][1,4]-diazepine; Figure 1) is an atypical antipsychotic medication used for the treatment of schizophrenic patients who are poor or nonresponders to traditional neuroleptic drugs (1, 2). It has been used to treat the positive as well as the negative symptoms of schizophrenia (3). Clozapine treatment is much less likely than phenothiazines and butyrophenones to cause extrapyramidal side effects. In the early 1970s, clozapine use was discontinued in therapeutical practice because of adverse effects such as reversible neutropenia which could progress to a potentially fatal agranulocytosis (4, 5). In the 1990s, clozapine was again made available in the United States in conjunction with constant monitoring of the white blood cell count (6–8) to avoid the onset of agranulocytosis.

Other important side effects, such as seizures, can occur during clozapine treatment (9); the epileptogenic effect of clozapine has been claimed to be dose-related and specifically correlated to high plasma clozapine levels (9). Because of the

incidence of adverse effects, clozapine must initially be administered at a low dose (25 mg once or twice a day). If tolerated, the dosage can gradually be increased in about 2 weeks to 200–400 mg/day. This dosage represents the mean daily dosage, and according to some authors (10) is the safest and most effective dosage. For poor responders, higher doses are needed (600–900 mg/day). Because it is necessary to control the amount of drug contained in the formulation, accurate and fast analytical methods are needed.

Numerous studies describing clozapine assays in plasma and in serum have been based on chromatographic methods such as gas chromatography (11–13), and particularly on liquid chromatographic (LC) techniques (14–20). Some studies also report the simultaneous determination of several drugs (including clozapine), based on the use of capillary electrophoresis (21–24). However, only a few reports on the dosage of clozapine in commercial preparations are presented in the literature (25–26).

Recently, we proposed a spectrophotometric method and an LC method with electrochemical detection (27) for clozapine determination in commercial tablets. We have now developed 3 new methods for the same purpose: a simple and sensitive LC method with UV detection, an accurate capillary zone electrophoresis (CZE) method, and a fast linear scan voltammetry (LSV) method.

Experimental

Reagents

(a) *Clozapine and dibenzepine*.—Kindly donated by Novartis Italia (Origgio, Milan, Italy).

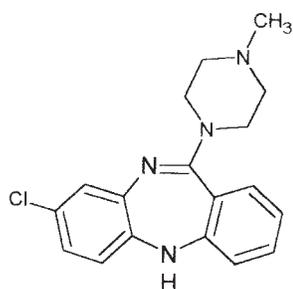
(b) *Loxapine*.—Donated by Lederle Laboratories (Pearl River, NY).

(c) *Methanol, acetonitrile, triethylamine, sodium hydroxide, and orthophosphoric acid (85%)*.—Analytical grade, produced by Carlo Erba (Milan, Italy).

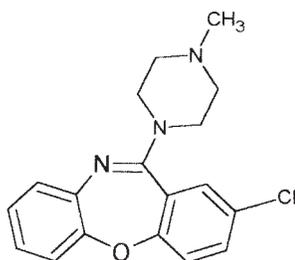
(d) *Polyvinylpyrrolidone (PVP 25)*.—From Serva (Heidelberg, Germany).

(e) *Ultrapure water*.—From a MilliQ apparatus by Millipore (Milford, MA) was used.

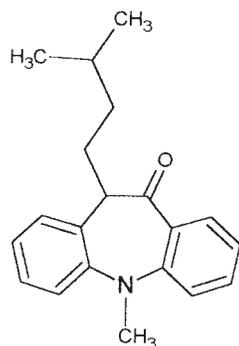
Pharmaceutical commercial preparations were examined; the only pharmaceutical preparation containing clozapine currently available in Italy is Leponex[®], produced by Novartis.



Clozapine



Loxapine



Dibenzepine

Figure 1. Chemical structure of analyte and internal standards.

The drug is available in 2 dosage forms: as tablets containing 25 and 100 mg of the drug.

The analyzed tablets contained 100 mg clozapine as the active ingredient, and magnesium stearate, silica, talcum, polyvinylpyrrolidone, corn starch, and lactose as excipients.

Apparatus and Experimental Conditions

The LC analysis (method A) was performed on a Beckman Instrument (Palo Alto, CA) Model 110B chromatographic pump (flow rate: 1.5 mL/min; loop: 20 μ L) and a Beckman Instrument Model 166 UV detector set at 230 nm. The stationary

phase was a Beckman Ultrasphere C₁₈ (250 \times 4.6 mm id, 5 μ m) reversed-phase column.

The mobile phase was composed of methanol, acetonitrile, and an aqueous solution containing 10.4mM phosphate buffer (19 + 15 + 66, v/v/v). Triethylamine was added to the buffer 0.40% (v/v) and the pH was brought to 1.9 with phosphoric acid. An isocratic elution was used for the determination of clozapine. Before use, the LC eluent was filtered through a 0.45 μ m filter (Millipore) and degassed by sonication. Dibenzepine was used as the internal standard.

The CZE analysis (Method B) was performed on a Bio-Rad (Hercules, CA) Biofocus 2000 Capillary Electrophoresis System with UV detection (245 nm). The separation was achieved by using an uncoated fused-silica capillary (total length 28 cm, effective length 19.4 cm), injecting the samples by pressure (10 psig) at the anodic end, and applying a potential of 15 kV. The background electrolyte (BGE) was a 25mM phosphate buffer, pH 2.5, containing 1.5% (w/v) polyvinylpyrrolidone (PVP). Loxapine was used as the internal standard.

The linear scan voltammetric analysis (LSV; method C) was performed on an AMEL (Milan, Italy) 433 voltammeter (solid graphite working electrode). The supporting electrolyte used was 10mM phosphate buffer, pH 2.5. The LSV method worked in oxidation at a +700 mV potential.

Solutions

Clozapine stock solutions for methods A and C and dibenzepine stock solutions for method A were prepared in methanol; clozapine stock solutions for method B were in 25mM phosphate buffer, pH 2.5. The standard working solutions were prepared by diluting suitable amounts of stock solution with methanol for the LC method (A); with ultrapure water for the CZE method (B); and with a 10mM phosphate buffer, pH 2.5, for the voltammetric method (C).

Leponex[®] solutions containing clozapine were prepared as follows. Twenty tablets were finely ground, and an amount of powder corresponding to 20 mg declared clozapine was then weighed and transferred into a test tube with 20 mL methanol for methods A and C, or 25mM phosphate buffer, pH 2.5, for method B. After agitation in an ultrasonic bath for 10 min, the supernatant was filtered to obtain a clozapine solution with a declared concentration of 1 mg/mL. To a known amount of this solution the internal standard (IS) solution (1 mg/mL) was added. The working solutions were prepared as described above for standard solutions.

Results and Discussion

Analytical Procedures

LC analysis.—As in our study of the determination of clozapine in the plasma of schizophrenic patients (28), based on an LC procedure with electrochemical detection, preliminary studies were performed using similar chromatographic conditions: a mobile phase constituted by acetonitrile, methanol, and a 10.4mM, pH 1.9, phosphate buffer (17.5 + 20 +

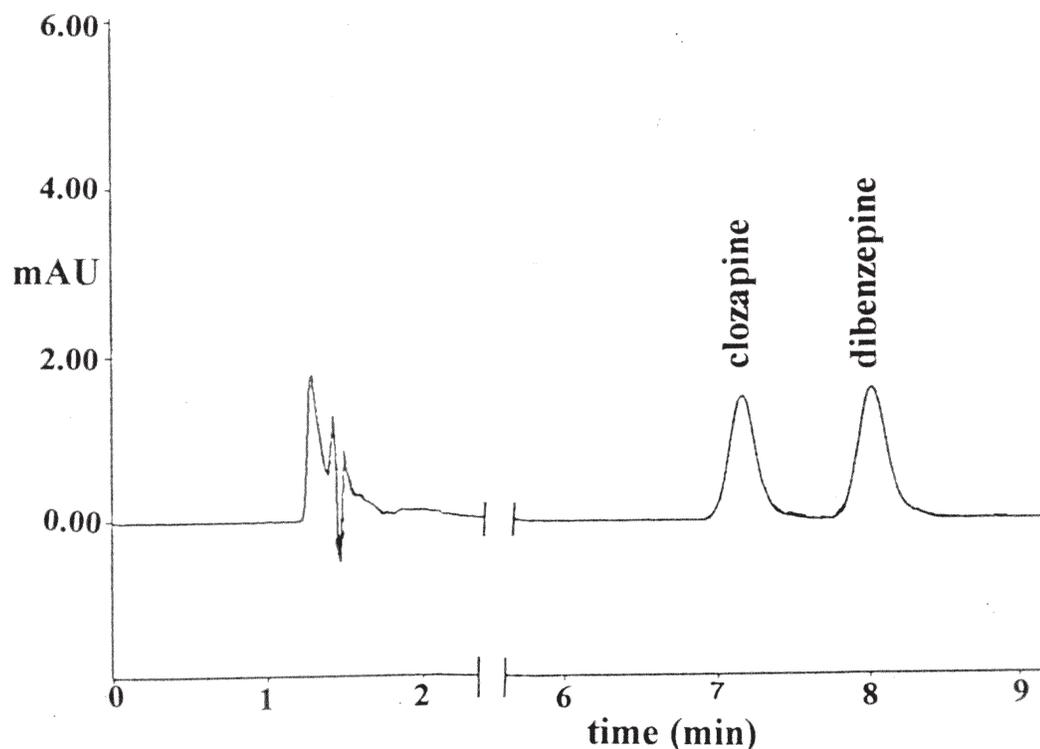


Figure 2. Chromatogram of 0.5 $\mu\text{g/mL}$ clozapine standard solution containing 0.5 $\mu\text{g/mL}$ IS (dibenzepine). Retention times: clozapine 7.1 min, dibenzepine 8.0 min.

Table 1. Characteristics of 3 analytical methods studied for quality control of clozapine

Parameter	Method		
	LC	CZE	LSV
Linearity range ($\mu\text{g/mL}$)	0.125–1.000	5–100	10–50
Straight line equation ^a ($Y = aX + b$):			
a	0.0019	0.0986	24.8230
b	0.0054	0.1046	10.4380
r	0.9998	0.9990	0.9990
Repeatability:			
RSD%	1.6	1.9	2.1
Concentration ($\mu\text{g/mL}$)	0.125	5	10
RSD%	1.4	1.4	1.9
Concentration ($\mu\text{g/mL}$)	0.500	50	25
RSD%	1.2	0.5	1.1
Concentration ($\mu\text{g/mL}$)	1.000	100	50
Intermediate precision:			
RSD%	1.9	2.0	2.2
Concentration ($\mu\text{g/mL}$)	0.125	5	10
RSD%	1.8	1.5	2.1
Concentration ($\mu\text{g/mL}$)	0.500	50	25
RSD%	1.6	0.9	1.9
Concentration ($\mu\text{g/mL}$)	1.000	100	50

^a Y = peak area, arbitrary units; X = clozapine concentration, ng/mL; r = correlation coefficient.

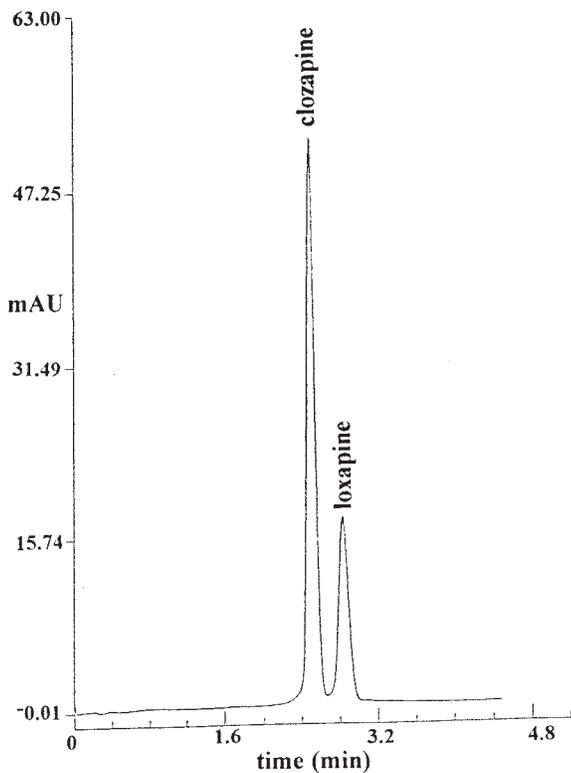


Figure 3. Electropherogram of 30 $\mu\text{g/mL}$ clozapine standard solution containing 30 $\mu\text{g/mL}$ IS (loxapine). Migration times: clozapine 2.4 min, loxapine 2.9 min.

62.5, v/v/v); and 0.25% (v/v) triethylamine, with a flow rate of 1 mL/min.

A UV detector was used ($\lambda = 230$ nm) instead of the amperometric detector, because it was more widely available, easier to use, and sufficiently sensitive for this analytical determination.

An IS was sought that would give more reliable results. Several drugs were tested; of these, paroxetine, carbamazepine, amitriptyline, loxapine, haloperidol, imipramine, ascorbic acid, glutathione, and methyl paraben were not detected within 15 min from the time of injection. Only dibenzepine, an antidepressant drug (Figure 1), was detected as a chromatographic peak with a retention time of 9.1 min which unfortunately was very near the clozapine peak. To enhance the resolution between clozapine and dibenzepine, the percentage ratio of the components of the mobile phase was modified. The optimal conditions were acetonitrile, methanol, and 10.4mM phosphate buffer, pH 1.9 (15 + 19 + 66 v/v/v).

Moreover, the flow rate was increased from 1 to 1.5 mL/min, to reduce analysis time. Figure 2 shows the chromatogram of a 0.5 $\mu\text{g/mL}$ clozapine standard solution containing also 0.5 $\mu\text{g/mL}$ dibenzepine. Under these optimal LC conditions, the peaks are fully resolved with retention times of 7.1 min for clozapine and 8.0 min for dibenzepine used as the IS.

The calibration curve was set up by plotting the value of the ratio between the area of clozapine and that of the IS

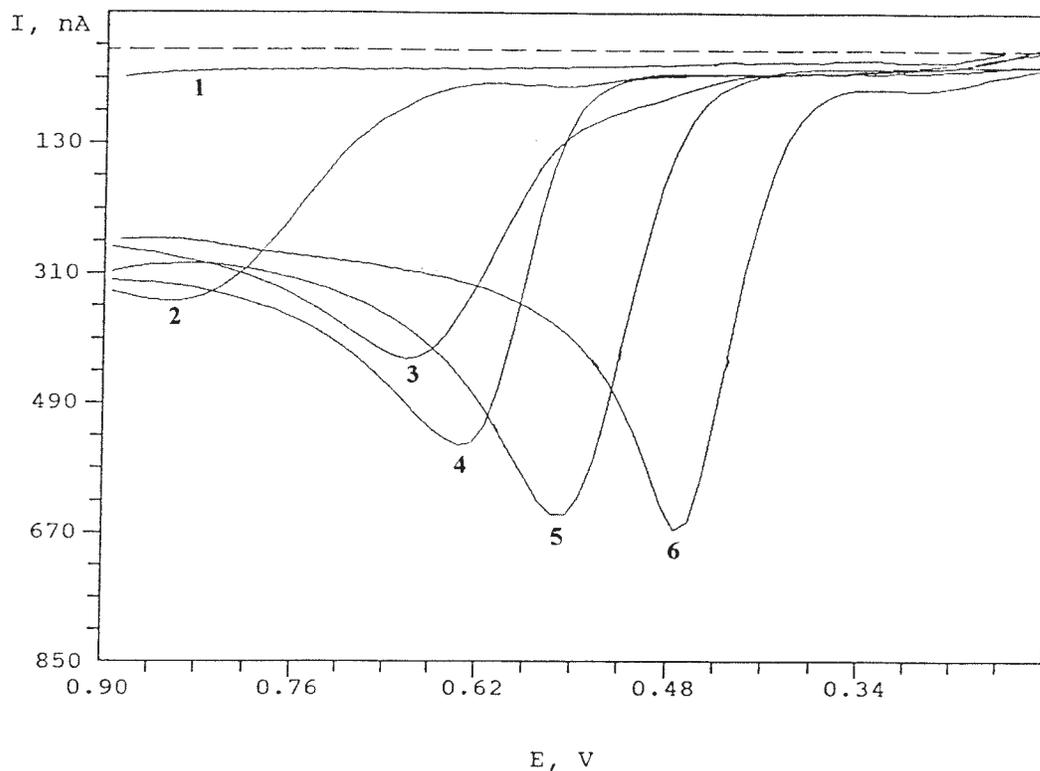


Figure 4. Voltammograms of (1) the supporting electrolyte (10mM phosphate buffer, pH 2.5) and 25 $\mu\text{g/mL}$ clozapine standard solution in phosphate buffer at different pH values: (2) 1.0; (3) 2.5; (4) 3.0; (5) 5.0; (6) 7.0.

Table 2. Clozapine assays in commercial tablets

Parameter	Method		
	LC	CZE	LSV
% Found of declared	100.5	100.4	98.6
Repeatability:			
RSD%	1.3	0.9	2.0
Concentration ($\mu\text{g/mL}$)	0.5	30	25
Intermediate precision:			
RSD%	1.8	1.4	2.2
Concentration ($\mu\text{g/mL}$)	0.5	30	25

(dibenzepine) against the clozapine concentration. Good linearity was found in the 0.125–1.000 $\mu\text{g/mL}$ range and the values of the repeatability and the intermediate precision were satisfactory (Table 1).

CZE analysis.—Preliminary assays were performed using a 50mM phosphate buffer, pH 2.5, an uncoated fused-silica capillary (total length 28 cm, effective length 19.4 cm), a voltage of 15 kV, and a UV detection at 206 nm. The short capillary led to high currents during the separation runs; for this reason, the total ionic concentration of the BGE was reduced from 50 to 25mM, to minimize the negative effects of the current.

Dibenzepine, which was used as the IS for the LC method, was discarded because its peak completely overlapped that of clozapine. We investigated loxapine as an alternative IS because its chemical structure is very similar to that of clozapine (Figure 1). Unfortunately, with the less concentrated buffer, the migration times of analytes are reduced (mobility is increased) and, thus, clozapine and loxapine are not fully baseline resolved. To obtain a complete separation of the compounds, PVP was added to the BGE. In fact, PVP is largely used as an additive because it can introduce a specific interaction with sample components and is used as a pseudo-stationary phase (29, 30). The addition of PVP to the BGE decreases the electrophoretic mobilities of the analytes, depending on their respective chemical properties. The different decreases

in mobility can enhance separation of the analytes and a higher selectivity of the method.

To use PVP, the UV detection wavelength had to be changed from 206 to 245 nm, because PVP has a considerable UV absorbance at 206 nm, which could cause severe interference in clozapine determination.

In an electropherogram of a standard solution containing 30 $\mu\text{g/mL}$ clozapine and 30 $\mu\text{g/mL}$ loxapine as the IS (Figure 3), the peaks are fully resolved with migration times of 2.4 and 2.9 min, respectively.

The calibration curve was set up by plotting the value of the ratio between the area of clozapine and that of the IS (loxapine) against the clozapine concentration. Good linearity was found in the 5–100 $\mu\text{g/mL}$ range. The data obtained from the repeatability and intermediate precision studies are shown in Table 1.

LSV analysis.—Clozapine is an electroactive substance which can be easily oxidized and is, thus, suitable for analysis with electrochemical techniques, such as linear scan voltammetry (LSV). To find the optimal leading conditions for the voltammetric analysis of clozapine, the pH of the supporting electrolyte, 10mM phosphate buffer, was varied between 1 and 7. Figure 4 shows the voltammograms of a 25 $\mu\text{g/mL}$ clozapine standard solution in a phosphate buffer recorded at different pH values (pH 1, 2.5, 3.0, 5.0, 7.0), and a blank of 10mM, phosphate buffer, pH 2.5. A comparison of these preliminary analyses showed that the intensity of the voltammetric peak was highest in the 5–7 pH range, and that the oxidation wave shifted toward lower potential values as the value of the buffer pH increased. Thus, as shown in the literature (31, 32), clozapine is more easily oxidized at higher pH values and is, thus, more unstable. In fact, at these pH values the determination of clozapine is difficult because the reproducibility is rather poor; therefore, the use of buffers at pH < 4 is necessary to obtain reliable results. All the measurements were performed using a pH 2.5 buffer to obtain satisfactory precision despite a lower sensitivity which is not needed for this kind of analysis. The calibration curve was set up by plotting the maximum current intensity value (+700 mV) against the clozapine concentration. Good linearity was found in the 10–50 $\mu\text{g/mL}$ range, and the parameters of repeatability and intermediate precision were also quite good (Table 1).

Table 3. Accuracy of assays for clozapine in tablets

Added clozapine conc.	Mean recovery (%) and repeatability					
	LC	RSD%	CZE	RSD%	LSV	RSD%
Low	98	2.2	101	1.2	101	2.2
		0.125 $\mu\text{g/mL}$		10 $\mu\text{g/mL}$		5 $\mu\text{g/mL}$
Middle	101	1.9	100	1.3	101	2.1
		0.250 $\mu\text{g/mL}$		20 $\mu\text{g/mL}$		15 $\mu\text{g/mL}$
High	102	1.8	100	0.9	102	1.8
		0.500 $\mu\text{g/mL}$		30 $\mu\text{g/mL}$		25 $\mu\text{g/mL}$

The validation data obtained from the analysis of standard solutions of clozapine by means of the 3 analytical methods are summarized in Table 1. The parameters of the regression equation relative to the different calibration curves (obtained by the least squares method) show good linearity, with correlation coefficients >0.9990 . The repeatability (RSD% intraday) and the intermediate precision (RSD% interday) values were obtained by analyzing clozapine solutions with concentrations of 0.125, 0.500, and 1.000 $\mu\text{g/mL}$ (method A); 5, 50, and 100 $\mu\text{g/mL}$ (method B); and 10, 25, and 50 $\mu\text{g/mL}$ (method C), $n = 6$. The relative standard deviation (RSD%) values were always $<2.2\%$.

Application to Pharmaceutical Formulations

Three methods: the LC (method A); CZE (method B); and voltammetric (method C) were studied and applied to the determination of clozapine in Leponex[®] tablets. The extraction procedure of clozapine from commercial tablets and the overall sample treatment consisted of a simple one-step extraction, filtration, and dilution (with methanol for methods A and C, and ultrapure water for method B). The sample treatment was very advantageous; in fact, efficiency and selectivity were good, and no interference from excipients was detected with any of the methods.

The data obtained for the quantitation of clozapine in tablets of Leponex[®] (summarized in Table 2) show that analyses of clozapine by the 3 methods gave similar results.

The repeatability (RSD% intraday) and intermediate precision (RSD% interday) of the total analysis were in the same range as those of the standard solutions. The quantities of the drugs found were in accordance with the values claimed by the manufacturer. For each method, the limits prescribed by the U.S. Pharmacopoeia 2000 were fulfilled; in fact, the USP range for tablets is 85–115% of the declared content (33).

The accuracy of the method was evaluated by means of recovery studies, adding known quantities of the reference drug standard solution to a known amount of the pharmaceutical formulation extract. Clozapine amounts of 0.125, 0.250, and 0.500 $\mu\text{g/mL}$ were added for method A; 10, 20, and 30 $\mu\text{g/mL}$ were added for method B; 5, 15, and 25 $\mu\text{g/mL}$ were added for method C; and good recovery values were obtained, within the 98–102% range (Table 3). The overall precision of these accuracy assays (expressed by the RSD% values, which were $<2.2\%$, $n = 6$) is in the same order of magnitude as the determination of the drug content.

Conclusion

The 3 proposed methods are suitable for rapid and reliable determination of clozapine for the quality control of commercial tablets. The sample treatment is very rapid, consisting of a simple one-step extraction, filtration, and dilution. No interference from excipients was found. It is concluded that with regard to repeatability, intermediate precision, and accuracy, all 3 methods give similar and satisfactory results and seem to be suitable for the determination of clozapine in commercial tablets. In particular, the LC and CZE methods are more sensi-

tive and selective than the voltammetric method, which is nevertheless much less expensive and more rapid.

Acknowledgments

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