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Direct and fast capillary zone electrophoretic method for the determination of Gleevec and its main metabolite in human urine

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Abstract

A capillary zone electrophoretic (CZE) method was investigated for the determination of Gleevec and its main metabolite (*N*-demethylated piperazine derivative) in human urine using a fused-silica capillary (75 μm I.D.×60 cm total length, 10 cm effective length). The separation was performed with an hydrodynamic injection time of 10 s (0.5 p.s.i.) a voltage of −25 kV, a capillary temperature of 25 °C and a 100 m*M* phosphoric acid adjusted to pH 2 with the addition of triethanolamine. Under these conditions, the analysis takes about 5 min. A linear response over the 0.4–30.0 mg l^{-1} concentration range was investigated for two compounds. A dilution of the sample was the only step necessary before the electrophoresis analysis. Detection limits of 0.1 mg l^{-1} for Gleevec and its metabolite ($S/N = 3$) were obtained. The developed method is easy, rapid and sensitive and has been applied to determine Gleevec and its main metabolite in clinical urine samples.

Keywords: Gleevec; Imatinib mesylate

1. Introduction

Gleevec (imatinib mesylate) is a new chemotherapy drug indicated for the treatment of patients with chronic myeloid leukemia (CML) in blast crisis, accelerated phase, or in chronic phase after failure of interferon-α therapy. It is the first of its kind developed to fight cancer by turning off an enzyme that causes cells to become cancerous and multiply.

Recently, signal transduction inhibitor 571 (STI 571 or Gleevec) was approved by the Food and Drug Administration (FDA) in record time [1] for the treatment of patients at any of the three stages of CML.

Gleevec or imatinib mesylate, is chemically designated as 4-[(4-methyl-1-piperazinyl)methyl]-*N*-[4-methyl-3-{[4-(3-pyridinyl)-2-pyrimidinyl]amino}-phenyl]benzamide methanesulfonate (Fig. 1). Imatinib mesylate is a protein tyrosine kinase inhibitor, which inhibits the Bcr-Abl tyrosine kinase at the in vitro, cellular, and in vivo levels. The compound selectively inhibits proliferation and induces apoptosis in Bcr-Abl positive cell lines as well as fresh leukaemic cells from Philadelphia chromosome positive chronic myeloid leukaemia (CML) and acute lymphoid leukaemia (ALL) patients [2].

In addition, imatinib is an inhibitor of the receptor

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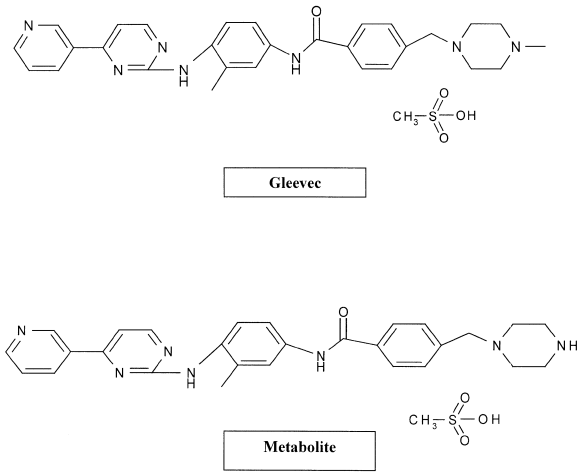

Fig. 1. Structures of Gleevec and its main metabolite.

tyrosine kinases for platelet-derived growth factor (PDGF) and stem cell factor (SCF), c-kit, and inhibits PDGF- and SCF-mediated cellular events. In vitro, imatinib inhibits proliferation and induces apoptosis in gastrointestinal stromal tumour (GIST) cells, which express an activating kit mutation.

The main circulating active metabolite in humans is the *N*-demethylated piperazine derivative, formed predominantly by CYP3A4. It shows in vitro potency similar to the parent imatinib. The plasma concentration for this metabolite is about 15% of the concentration for imatinib and the terminal half life is approximately 40 h at steady state. There is no change in the kinetics of this metabolite on repeated dosing and it did not unexpectedly accumulate after longterm administration of Gleevec to patients [3].

Based on the recovery of compounds after an oral ^{14}C-labelled dose of imatinib, approximately 81% of the dose was eliminated within 7 days in feces (68% of dose) and urine (13% of dose). Unchanged imatinib accounted for 25% of the dose (5% in urine, 20% in feces), the remainder being metabolites [4].

The recommended dosage of Gleevec is 400 mg per day for patients in chronic phase CML and 600 mg per day for patients in accelerated phase or blast crisis. The prescribed dose should be administered orally. Treatment should be continued as long as the patient continues to benefit. Dose decrease from 400 to 100 mg in patients with severe adverse drug reaction.

Liquid chromatography–tandem mass spectrometry has been demonstrated to be a useful technique for study and determination of the anti-leukemia drug (Gleevec) and its main metabolite in human plasma [5] and in monkey plasma using a semiautomated solid-phases extraction procedure [6].

In this paper, we propose a rapid and easy method using capillary zone electrophoresis (CZE) to determine Gleevec and its main metabolite (*N*-demethylated piperazine derivative) in urine without any treatment of the biological sample.

2. Materials and methods

2.1. Materials

All solvents and reagents were of analytical-reagent grade unless indicated otherwise. Gleevec and its main metabolite (*N*-demethylated piperazine derivative) were obtained from Novartis (Basel, Switzerland). Standard solutions were prepared with deionised water (Milli-Q quality) and stored at 4 °C.

Urine diluted solutions were daily prepared by diluting the freshly human urine with purified water (1:1, v/v).

Buffer solutions were prepared by dissolving the adequate quantity of H_3PO_4 in deionised water and the adjusting with triethanolamine to the required pH.

The set of separation vials was changed after six runs.

2.2. Apparatus and operating conditions

A Beckman P/ACE system MDQ (Fullerton, CA, USA) equipped with a diode-array detection system was used. Beckman capillary electrophoresis software controlled the system. Separation was carried out on a 60 cm long (10 cm to the detector, short way)×75 μm I.D. fused-silica capillary housed in a cartridge with a detector window 800×100 μm. The wavelength selected for the electropherograms was 257 nm. The capillary was conditioned prior to its use by rising with 0.5 *M* NaOH for 20 min, water for 10 min and finally phosphate buffer. At the start of each sequence, the capillary was washed with the separation buffer. All separations were conducted using 2-ml vials for rinsing and washing and 200-μl

polypropylene vials as sample vials. Biological samples were preserved at 25 °C inside the capillary electrophoretic equipment.

2.3. Procedure

The capillary—75 μm I.D.×60 cm (total length), 10 cm (effective length)—was filled with separation buffer for 2 min (20 p.s.i.; 1 p.s.i.=6894.76 Pa), followed by a 10 s hydrodynamic injection (0.5 p.s.i., using the short way to capillary) of the diluted urine samples (water:urine). The separation was performed at −25 kV (reversed polarity mode) for 5 min (with a −83.3 kV min^{-1} ramp voltage). Under the selected conditions the current was −69.4 μA. Due to the effective length of capillary is 10 cm, the total capillary length can be reduced to 31 cm that is the shortest length that Beckman P/ACE System MDQ offers. In this case, the injection time and the separation voltage should be modified to obtain the same volume the sample injected and electric field mentioned above, respectively. In all cases the electropherograms were recorded at 257 nm. Triplicate injections of the solutions were performed and average-corrected peak areas (CPA) (area/migration time) were used for quantitative analysis. The data generated from the first two injections of a sequence were not used on account of the necessary equilibration system.

3. Results and discussion

3.1. Preliminary investigations

Preliminary experiments by injection from the long way of the capillary to the window (50 cm as effective length) of the solution 2.4 mg l^{-1} of Gleevec and 1.5 mg l^{-1} of metabolite showed that the compounds could be determined in 9 min (Fig. 2), with a very good resolution, but poor sensitivity, probably due to the longitudinal molecular diffusion of sample components. For this reason, the injection from the short way of the capillary (10 cm as effective length) was tried with the object to get more sensitive in less time of analysis. Fig. 2 shows that a sensitive and good separation of peaks was obtained by this injection method. The injection was done by the short way in all the experiments.

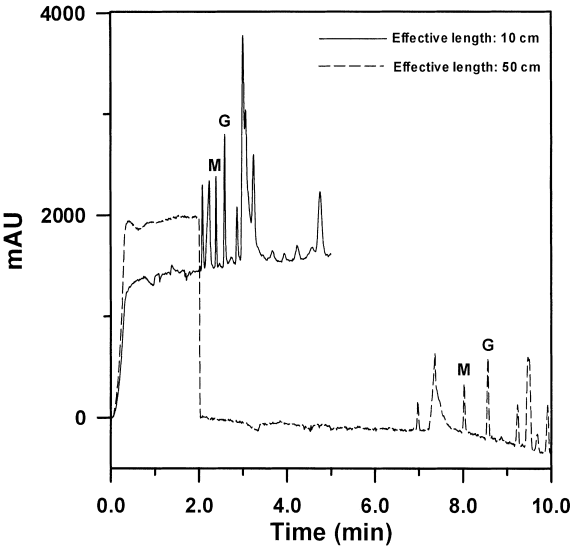

Fig. 2. Influence of the effective capillary in the determination of the solution 2.4 mg l^{-1} of Gleevec (G) and 1.5 mg l^{-1} of metabolite (*M*). Operating conditions: 100 m*M* of phosphate buffer, 25 kV of voltage, 25 °C capillary temperature, 5 s injection time (0.5 p.s.i.).

3.2. Effect of pH

The pH of the running electrolyte had a significant impact on the ionisation of the acidic silanols of the capillary wall and on the electrophoretic mobilities of the compounds studied. On account of the structure of the analytes (Fig. 1), basic or acid buffers could be used to promote their ionisation. Some experiments were carried out in this way in order to evaluate the influence of pH on Gleevec and its metabolite separation. The buffer tested was phosphate (20 m*M*). A pH range of 2–12 was tested. In acid medium (pH<4), Gleevec and its metabolite migration times were shorter than the EOF. At these low pH values high migration times of EOF were found—more than 10 min for pH 2 and 3, using 20 m*M* phosphate and 25 kV voltage. At pH values between 5 and 12, the compounds have the same migration time as the EOF. This shows that the two drugs are in the cationic form at <5 and as nonionic form at pH 5–12. In order to take CZE advantage, an

acid pH 2.0 (adjusted by the addition of triethanolamine to a solution of phosphoric acid) was selected for the study and direct determination of the two mentioned drugs in urine, because higher pH values gave a very poor resolution between the two compounds in the biological fluid (urine).

3.3. Influence of phosphate buffer concentration

The effect of the concentration of buffer solution (60–140 m*M*) on the migration time of the compounds was studied (Fig. 3). When the concentration of buffer increases the migration time remains constant but the current intensity increases in proportion to the concentration of the separation electrolyte. The resolution between Gleevec and its metabolite is better when the concentration of the buffer increases from 60 to 120 m*M*. For higher concentrations the resolution stayed constant due to a increment in the temperature of the capillary caused by the Joule effect. A buffer concentration of 100 m*M* was selected to maintain a good peak shape and a low current in order to minimise the noise and baseline aberrations. Under these conditions the migration times for Gleevec and its metabolite were 2.22 and 2.74 min, respectively.

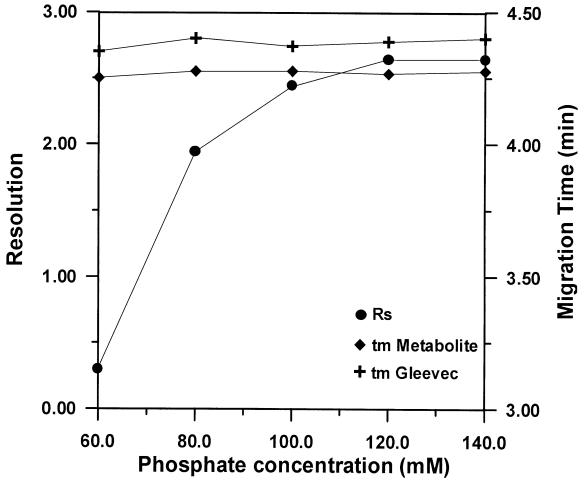

Fig. 3. Influence of concentration of phosphate on migration times and resolution. Operating conditions: 100 m*M* phosphate buffer, pH 2; 25 kV as separation voltage; 25 °C capillary temperature, 10 s injection time (0.5 p.s.i.).

3.4. Influence of voltage

The effect of varying the voltage from −5 to −30 kV was investigated under the conditions selected above (Fig. 4). A potential of −25 kV yielded the best compromise in terms of run time, current regenerated and efficiency of separation. Therefore this potential was used in subsequent stages of the method development.

3.5. Effect of temperature

The effect of temperature on separation was investigated in the range 20–35 °C. A decrease in temperature resulted in increased migration times of the solutes due to higher electrolyte viscosity. The selectivity of separation was slightly affected; 25 °C was selected as it gives the best compromise between resolution and run time with an acceptable level of baseline noise.

3.6. Optimisation of injection times

In order to decrease the detection limits in urine, the injection time (0.5 p.s.i.) was varied between 3 and 15 s always using the short way to window of capillary. The corrected area increased with longer injection times but we also got poor resolution between the two peaks. As could be expected when

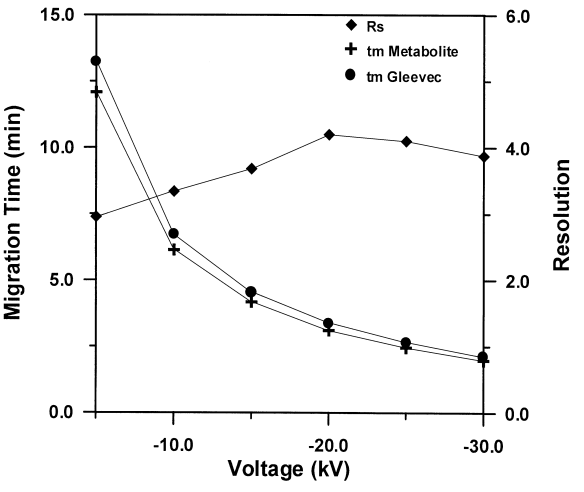

Fig. 4. Influence of separation voltage on migration times and resolution. Operating conditions as in Fig. 2.

the short way of capillary is used (10 cm), the time of injection are very limited in values, due to the big plug sample length introduced into the capillary, that must be lower than 2% of the total effective capillary length. In our case, a 10 s injection time (9.7% of total effective capillary length) gave a reasonable resolution between the two drugs with a good peak intensity. For injection times up to 10 s poor resolution was found between this metabolite and the urine compounds present in the sample. For this reason the optimum value was chosen as 10 s. The specificity of method versus endogenous components of the matrix were assessed using five different pools of urine samples (three from women and two from men).

3.7. Selected conditions

From the studies carried out before, the procedure summarised below was convenient to separate two compounds in diluted urine samples; a fused-silica capillary of 60 cm×75 μm I.D., 100 m*M* phosphate buffer (pH 2) as electrolyte of separation; 25 °C as capillary temperature, −25 kV (−83.3 kV min^{-1} in 0.3 min as voltage ramp) and a detection window of 800×100 μm. The selected wavelength was 257 nm for Gleevec and its metabolite. Under these conditions, the migration times were 2.29 and 2.45 min for Gleevec and its metabolite, respectively.

The obtained electropherogram under the selected conditions is presented in Fig. 5.

In all cases the urine samples were diluted with deionised water in a 1:1 ratio. This was the only step necessary before the analysis.

3.8. Quantitative aspects

3.8.1. Limits of detection and quantitation

Limits of detection (LODs) and quantification (LOQs) were estimated in the usual way. The LOD was obtained as the drug concentration that caused a peak with a height three times the baseline noise level and the LOQ was calculated as 10 times the baseline noise level [7].

The LODs were about 0.1 mg l^{-1} for Gleevec and its metabolite ($S/N = 3$) and the LOQs was estimated to be about 0.3 mg l^{-1} in both types of samples (refrigerated and frozen urine).

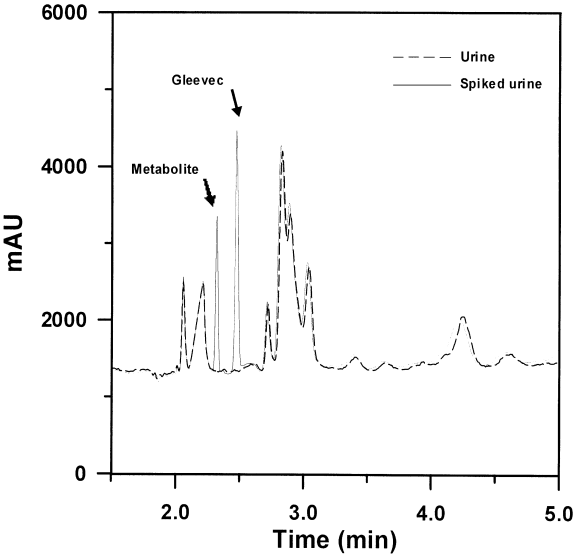

Fig. 5. CZE electropherograms of a drug-free urine and a urine spiked with 1.5 mg l^{-1} of metabolite and 2.4 mg l^{-1} of Gleevec. Optimise operating conditions: 100 m*M* phosphate buffer, pH 2, −25 kV as separation voltage; 25 °C capillary temperature, 10 s injection time (0.5 p.s.i.).

3.8.2. Linearity range and calibration curves

All results were obtained by using CPA for calculations (to obtain CPA peak area was divided by its corresponding migration time) [7].

The linearity of the assay was checked by injecting diluted urine (water–urine, 1:1) solutions (men and women) spiked with two compounds in the concentration range from 0.4 to 30.0 mg l^{-1} ($n = 7$) the regression lines, calculated using least squares method, were

Male fresh urine

$$\text{Metabolite: CPA} = 37.40 \pm (572.78) + 1065.17 \pm (43.07)c \qquad r^2 = 0.9988 \tag{1}$$

$$\text{Gleevec: CPA} = -16.76 \pm (458.14) + 1105.51 \pm (35.96)c \qquad r^2 = 0.9992 \tag{2}$$

Female fresh urine

$$\text{Metabolite: CPA} = -108.08 \pm (277.57) + 1086.64 \pm (20.87)c \quad r^2 = 0.9997 \tag{3}$$

$$\text{Gleevec: CPA} = -32.01 \pm (165.89) + 1089.32 \pm (13.02)c \quad r^2 = 0.9999 \tag{4}$$

Unfrozen male urine

$$\text{Metabolite: CPA} = 6.18 \pm (462.41) + 1143.81 \pm (34.77)c \quad r^2 = 0.9993 \tag{5}$$

$$\text{Gleevec: CPA} = -153.39 \pm (552.29) + 1182.13 \pm (43.34)c \quad r^2 = 0.9999 \tag{6}$$

where c represents concentrations of standard solutions (mg/l) and r^2 denotes the determination coefficient, with the confidence intervals calculated at $\alpha = 0.05$.

An analysis of variance (ANOVA) test was performed to compare the different regression lines obtained, to determine whether the data could be combined to enable estimation of the appropriate quantities by use of a comprehensive regression line [8,9]. The ANOVA values are shown in Table 1. If the experimental value of F is less than the theoretical value there are no significant differences between variances whereas if F_{theor} is less than the experimental value of F there are significant differences between variances. The t test was carried out to compare the different slopes of the calibration graphs. If the experimental value of t is less than the theoretical value there are no significant between slopes.

No significant differences were found between the calibration graphs performed in male and female urine but significant differences where found between fresh urine (taken directly from a healthy man or woman) and frozen and unfrozen (thawed) urine (urine stored for 2 weeks at −18 °C and thawed the day of the analysis) (Table 1). This could be due to changes in the matrix composition (change of ionic strength) after the freeze–thaw process in the urine. The high value of F experimental between the (2) and (4) calibration graphs is due to the estimated variance of the residual for the two above calibration. In this case the t-test must not be done with a global estimated variance for the two slopes. The individual variance of the two slopes must be considered.

3.9. Repeatability and reproducibility

Two different samples containing 0.6 and 0.4 mg l^{-1}, Gleevec and its metabolite, respectively, were prepared and analysed on 2 different days using the optimised method. Repeatability was studied by performing a series of nine separations of one of these samples. The reproducibility was studied by performing nine separations of other sample 24 h later than analysis of the first, under the same conditions. The results showed that the repeatability (using CPA) for two compounds on each day is satisfactory (Table 2). The comparison of averages with the Snedecor test did not provide any significant

Table 1
Analysis of variance of regression

Regression lines	F_{exp}	F_{theor}	t_{exp}	t_{theor}
(1) and (3)	4.26	5.05	1.15	2.57
(2) and (4)	7.63	5.05	1.09	2.57
(1) and (5)	1.53	5.05	3.65	2.57
(2) and (6)	1.45	5.05	3.50	2.57

Table 2
Repeatability and reproducibility

	Gleevec		Metabolite	
	Day 1	Day 2	Day 1	Day 2
$\text{CPA}_{\text{(mean)}}$	373	372	212	203
SD	20.68	18.41	17.03	9.34
RSD (%)	5.54	4.95	8.03	4.59
F_{exp}	1.26		3.32	
F_{Theor}	3.44		3.44	

Table 3
Recoveries obtained in different spiked urines

Sample	Gleevec		Metabolite	
	Added (mg l^{-1})	Recovery (%)	Added (mg l^{-1})	Recovery (%)
S1	7.3	100	3.8	104
S2	9.5	100	4.8	102

difference between the two days series, for α (0.05, $n=9$) [7].

3.10. Applications

To demonstrate the usefulness of the proposed method, several aliquots of Gleevec and its metabolite standard solutions were added to the spiked urine (Table 3). Urine samples from two patients undergoing medical treatment with Gleevec (real samples) were also analysed (frozen samples).

Fig. 6 shows the electropherogram corresponding to the urine sample of patient A undergoing medical treatment with Gleevec (100 mg/day), after dilution with water to have a concentration of the drugs in the range examined. A good resolution was obtained between the interfering compounds of the matrix and the analysed drugs. In order to evaluate the matrix effect and because there were significant differences between the calibration graphs, the analysis was performed in fresh and frozen urines. In all cases the standard addition method was used for the determination of drugs in real samples. The urine sample of patient A undergoing medical treatment with Gleevec (100 mg/day) was diluted 1:3 with water and the concentrations found using this method were 6.9 and 3.1 mg l^{-1} of Gleevec and metabolite, respectively. However, in the urine sample of patient B (600 mg/day) it was necessary to dilute with water in the ratio (urine–water) of 1:8; in this case the concentrations found were 34.2 and 14.3 mg l^{-1}, of Gleevec and metabolite, respectively. In all cases, application of the *t*-test for the slopes of the calibration graphs showed no statistically significant differences, with regard to the obtained calibration graphs in unfrozen urine. The two linear calibrations (5) and (6) could be proposed as possible calibration graphs. There is therefore no evidence of systematic error affecting the determination of Gleevec and its metabolite in urine by the proposed method. In all cases, triplicate samples were used for each determination.

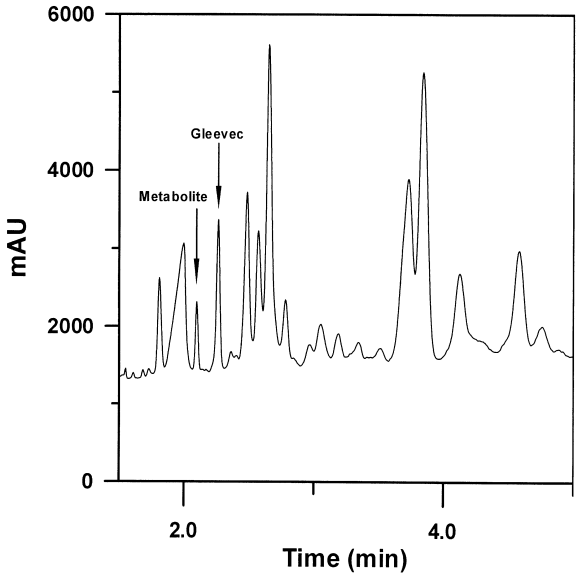

Fig. 6. CZE electropherogram of urine sample (water–urine, 1:3) from patient A (dosages Gleevec 100 mg per day). Operating conditions as in Fig. 5.

4. Conclusions

In this work, a rapid, easy and sensitive method for the determination of Gleevec and its main metabolite in urine by CZE is described. The detection limits obtained are low enough to determine these compounds in clinical samples. As a consequence we conclude that the proposed CZE method could be an alternative for the determination of these drugs in clinical samples. The proposed method is easier and faster than those previously proposed for the determination of the two drugs. The linearity, recovery, precision and sensitivity were highly satisfactory.

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