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Quantitative Determination of Amitriptyline and Its Metabolite in Rat Plasma by Liquid Chromatography-tandem Mass Spectrometry

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A rapid, specific, and reliable LC-MS/MS-based bioanalytical method was developed and validated in rat plasma for the simultaneous quantitation of amitriptyline and its metabolite nortriptyline. Chromatographic separation of these analytes was achieved on a Gemini C18 column (50 × 4.60 mm, 5 μm) using reversed-phase chromatography. The mobile phase was an isocratic solvent system consisting of 1% formic acid in water and methanol (10:90, v/v), at a flow rate of 0.2 mL/min. The analytical range was set as 0.1-500 ng/mL for amitriptyline and 0.08-500 ng/mL for nortriptyline using a 200 μL plasma sample. The accuracy and precision of the assay were in accordance with FDA regulations for the validation of bioanalytical methods. The validated method was successfully applied to a pharmacokinetic study in six rats after oral administration of amitriptyline (15 mg/kg). This method allows laboratory scientists to rapidly determine amitriptyline and nortriptyline concentrations in plasma.

Key Words : Amitriptyline, Nortriptyline, LC-MS/MS, Quantitation, Pharmacokinetics

Introduction

Amitriptyline is a typical tricyclic antidepressant (TCA) used for the treatment of major depression since the 1960s. It induces a specific pharmacodynamic effect primarily by blocking presynaptic uptake of amines (norepinephrine, dopamine, and serotonin). Amitriptyline metabolism involves hepatic microsomal enzymes (mainly CYP2C19 and CYP3A4) that demethylate the aliphatic side chain, generating the pharmacologically active metabolite nortriptyline.[1] Amitriptyline has a relatively narrow therapeutic index and overdosing can lead to severe poisoning including cardiovascular, respiratory, and central nervous system toxicity. TCA overdose is a primary cause of severe poisoning in many hospitalized patients and an effective treatment has yet to be identified.

To date, various assays for amitriptyline and its metabolites in biological samples have been reported. These are mainly based on reversed-phase separation followed by ultraviolet[2-4] or particle beam mass spectrometric determination.[5] However, the sensitivity of these methods is low and requires a large sample volume. Kollroser and Schober described a liquid chromatography (LC) with tandem mass spectrometry (MS/MS) method with increased sensitivity.[6] However, it required 1 mL plasma aliquots to reach the lower quantitation limit. A recent study by described a liquid-liquid extraction (LLE) and solid-phase extraction (SPE) method improved sensitivity.[7,8] These reported methods have still higher quantification limits than our developed method.

In our study (in rats), large sample volumes were not available; thus, we developed a simple and sensitive method for extracting and determining amitriptyline and nortriptyline concentrations in plasma. These present method was fully validated and applied to characterize the time course of plasma amitriptyline and its metabolites concentrations following oral administration in rats.

Materials and Methods

Chemicals. Amitriptyline, nortriptyline, and the internal standard (IS) acetaminophen were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). All other analytical grade chemicals and solvents were purchased from Duksan Pure Chemical (Ansan, Republic of Korea). A PURELAB Ultra system from ELGA (Marlow, UK) was used in the laboratory to produce deionized water.

Instrumentation. Plasma concentrations of amitriptyline and nortriptyline were quantitated by LC-MS/MS using a PE SCIEX API2000 (triple-quadrupole) system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface. The analytical data were processed by Analyst 1.4.1 software (Applied Biosystems).

Liquid Chromatographic Conditions. Chromatographic separation was achieved on a Gemini C18 column (50 × 4.60 mm, 5 μm; Phenomenex, Torrance, CA, USA). The column temperature was maintained at 25 °C. The mobile phase was an isocratic solvent system consisting of 1% formic acid in water and MeOH (10:90, v/v) at a flow rate of 0.2 mL/min.

Mass Spectrometric Conditions. The mass spectrometer

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was operated in the positive ion mode. The instrument parameters for monitoring amitriptyline, nortriptyline, and the IS during method validation and sample analysis were as follows: TurboIonSpray (TIS) temperature of 350 °C, exhausting gas pressure of 45 psi, nebulizing gas pressure of 95 psi, curtain gas pressure of 50 psi, declustering potentials (DP) of 21 V, 26 V, and 21 V, respectively, entrance potential (EP) of 12 V, and collision energies (CE) of 39 eV, 33 eV, and 25 eV for amitriptyline, nortriptyline, and the IS, respectively. The following precursors to product ion transitions were used in the multiple reactions monitoring (MRM) of amitriptyline, nortriptyline, and the IS, respectively: *m/z* 278→90, *m/z* 264→90, and *m/z* 152→110, with dwell times of 250 ms. The mass spectrometer was operated at unit mass resolution for both the first and third quadrupoles.

Preparation of Standard and Quality Control (QC) Samples. Standard stock solutions containing 1 mg/mL concentrations of free-form amitriptyline, nortriptyline, and the IS were made in methanol. All standard stock solutions were stored at −20 °C. A series of working solutions were obtained by diluting appropriate amounts of these standards with 100% methanol to six different concentrations. Working solutions were stored at 4 °C in the dark.

The IS was prepared in methanol at 0.5 μg/mL. Calibration standards and QC samples were prepared by spiking 20 μL of the working solutions and 200 μL of the IS into 160 μL of drug-free rat plasma. The resulting plasma concentrations were 0.1, 0.8, 4, 20, 100, and 500 ng/mL for amitriptyline and 0.08, 0.8, 4, 20, 100, and 500 ng/mL for nortriptyline. QC samples were prepared in blank rat plasma at three levels: low (0.5 ng/mL), middle (10 ng/mL), and high (250 ng/mL) for amitriptyline, and low (0.5 ng/mL), middle (10 ng/mL), and high (250 ng/mL) for nortriptyline. All QC samples were stored at −70 °C.

Sample Preparation. A 200-μL aliquot of rat plasma was mixed with 200 μL of IS working solution (0.5 μg/mL) prior to extraction with 3 mL ethyl acetate by vortex shaking for 5 min. Following centrifugation at 3,000 rpm for 10 min, the organic layer was transferred to another tube and evaporated at 40 °C under a gentle stream of nitrogen. The dry residue was then reconstituted with 200 μL MeOH and vortex-mixed for 30 s. A 10-μL solution was injected into the HPLC-MS/MS.

Method Validation. The method was validated with respect to selectivity, linearity, accuracy, precision, percent recovery, matrix effect, and stability.

Calibration curves were constructed between 0.1 and 500 ng/mL for amitriptyline and between 0.08 and 500 ng/mL for nortriptyline by determining the best fit of analyte peak area ratios to the IS (y) as a function of nominal concentration (x). The data were fitted to the equation $y = bx + a$ using a $1/x^2$ weighted least-squares regression. QC and plasma sample concentrations were calculated based on the calibration curves. Intra- and inter-day precision and accuracy were evaluated by assaying six replicates of each spiked QC sample at the low, middle, and high concentrations on 5 separate days. Precision is expressed as a relative standard deviation (RSD). Accuracy was calculated as the percent error in the calculated mean concentration relative to the nominal concentrations (RE).[9] For the assay to be considered acceptable, the precision and accuracy at each QC level had to be within 15%. Absolute recoveries at low, middle, and high plasma concentrations were determined in triplicate by comparing the analyte peak area in spiked post-extraction plasma with the corresponding concentration in the spiked sample. Matrix effects were investigated by comparing the extraction samples of blank plasma from six different drug-free rats spiked with low, middle, and high concentrations of QC followed by direct injection of the mobile phase spiked with the analytes. Stability under the experimental conditions was investigated at low and high QC concentrations. Short-term, post-extraction, freeze-thaw, and long-term stabilities were assessed.[10] Short-term storage stabilities of analytes after processing were evaluated by testing their stabilities after extraction and storage for 6 h at room temperature. Long-term stability was examined for 20 days at −70 °C. Freeze-thaw stability testing was determined after freezing at −70 °C and thawing to room temperature three times.

Animal Studies. Animal experiments were performed according to institutional guidelines for the care and the use of laboratory animals, and approved by the animal ethics committee of Chungnam National University. Six Sprague-Dawley (SD) rats weighing 230 ± 15 g (Orient Bio, Inc., Seongnam-si, Gyeonggi-Do, Republic of Korea) were housed in an animal facility at the College of Pharmacy, Chungnam National University. Before starting the experiments, animals were kept under standard laboratory conditions (12/12 h light/darkness, 22 ± 2 °C, 50-60% humidity) for at least 1 week. Following an overnight fast, amitriptyline was orally administered to all rats (15 mg/kg). Heparinized blood samples (600 μL) were collected from the ocular plexus venous of each rat 0.35, 1, 2, 4, 6, 12, 24, 36, and 48 h after dosing. The rats were allowed free access to water during the experiment to maintain normal body conditions. Plasma (200 μL) was immediately separated by centrifugation at 3,000 rpm for 10 min, then transferred to labeled tubes and stored at −70 °C until use.

Data Analysis. Pharmacokinetic analysis was performed using noncompartmental methods with WinNonlin standard version 2.1 software (Pharsight Corp., Palo Alto, CA, USA). The area under the plasma concentration-versus-time curve (AUC) was calculated using trapezoidal estimation and extrapolated to infinity. The plasma concentrations of amitriptyline and nortriptyline as a function of time were used to determine the maximum plasma concentration (C_{max}) and the time (T_{max}) required to reach C_{max}. An elimination rate constant (K_{el}) was obtained by linear regression of the terminal phase, and the elimination half-life ($t_{1/2}$) was calculated as $0.693/K_{el}$. The results are presented as the mean ± standard deviation.

Results and Discussion

Bioanalytical Method Development. Precursor ions for amitriptyline, nortriptyline, and their corresponding ions were identified and quantitated from spectra obtained after injection of standard solutions into a mass spectrometer with an electrospray ionization source. The system was operated in positive ionization mode with nitrogen collision gas in Q2 of a MS/MS system. Amitriptyline, nortriptyline, and the IS produced protonated ions at *m/z* 278, 264, and 152, respectively. Product ions were scanned in Q3 following collisions with nitrogen in Q2 at *m/z* 90, 90, and 110 for amitriptyline, nortriptyline, and the IS, respectively (Fig. 1).

Significant peak tailing was observed for theophylline and its metabolites when using an acetonitrile mobile phase. Therefore, several combinations of methanol and water were evaluated to sufficiently resolve each compound while minimizing both noise and peak tailing effects. We found that including 1% formic acid in the mobile phase improved the peak shape. The optimal mobile phase was identified as a 90:10 (v/v) mixture of MeOH and water with 1% formic acid. The retention times of amitriptyline, nortriptyline, and the IS in rat plasma were approximately 2.5-3 min, and the total run time for each sample was approximately 4 min.

Assay Specificity and Matrix Effect. Ion chromatograms from a blank sample (non-spiked blank plasma), a zero sample (spiked with the IS), a blank sample spiked at LOQ (0.1 ng/mL for amitriptyline, 0.08 ng/mL for nortriptyline), and samples containing a midrange concentration of the two analytes showed no significant interference peaks at the amitriptyline, nortriptyline, or IS retention times (Fig. 2).

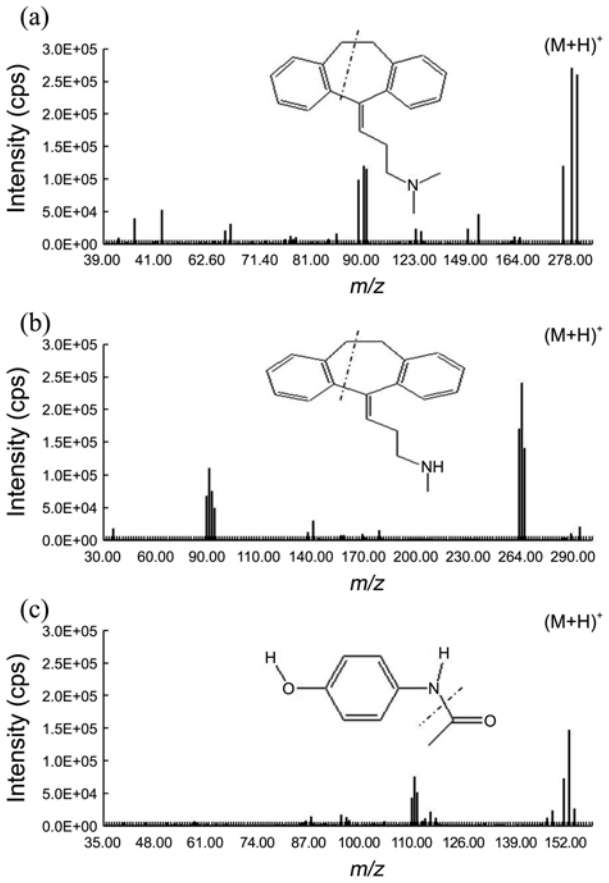

Figure 1. Tandem mass spectra showing ions from (a) amitriptyline, (b) nortriptyline, (c) acetaminophen (internal standard, IS) using electrospray ionization in positive ion mode.

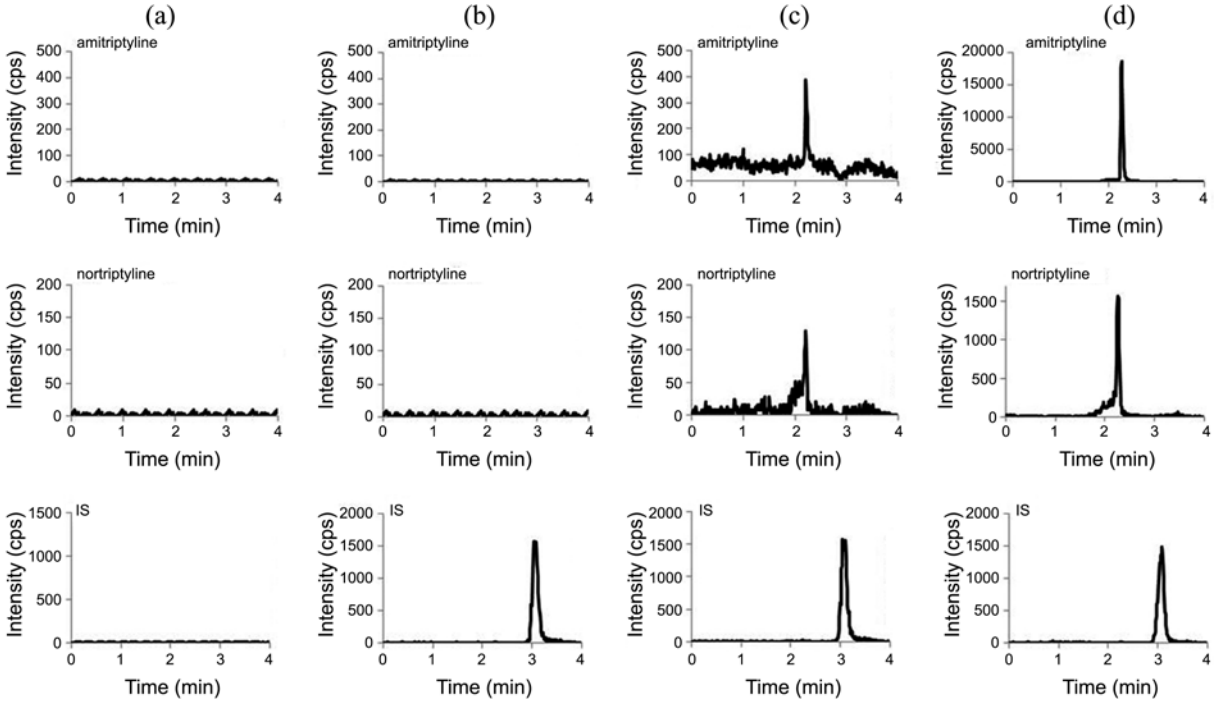

Figure 2. Representative MRM chromatograms of amitriptyline, nortriptyline and the IS in (a) SD rat blank plasma, (b) SD rat blank plasma spiked with the IS, (c) SD rat blank plasma spiked with LOQ (0.1 ng/mL for amitriptyline and 0.08 ng/mL for nortriptyline) with the IS, and (d) SD rat blank plasma with mid-concentration (10 ng/mL) analytes and the IS.

Table 1. Matrix effects and percent recoveries of amitriptyline, nortriptyline and the IS in rat plasma (n = 6)

Concentration (ng/mL)	Matrix effect (Mean % ± S.D.)	Recovery (Mean % ± S.D.)
Amitriptyline		
0.5	92.84 ± 4.12	94.21 ± 1.01
10	97.66 ± 1.54	101.11 ± 2.61
250	94.11 ± 1.40	97.51 ± 3.19
Nortriptyline		
0.5	97.64 ± 1.41	95.44 ± 2.22
10	89.63 ± 2.27	98.78 ± 5.14
250	91.21 ± 3.31	97.61 ± 1.27
I.S		
50	94.36 ± 2.77	–
500	97.96 ± 1.98	–
5,000	96.67 ± 2.17	–

Matuszewski outlined the importance of evaluating the matrix effect in any LC-MS/MS method.[11] In our study, the matrix effect was assessed as follows: analytes were added at three concentrations (0.5, 10, 250 ng/mL for amitriptyline, and 0.5, 10, 250 ng/mL for nortriptyline) to the blank matrix from five different individuals (15 total). These samples were then subjected to the analytical procedure and compared with the standard working solutions. The results indicated that co-elution of endogenous species did not interfere with IS ionization analytes (Table 1). Thus, these chromatographic conditions provided adequate separation between the solvent front and the analytes, which may also have reduced the risk of ion suppression.

Linearity of Calibration Curves and the Lower Limit of Quantitation. The calibration curves were linear from 0.1 to 500 ng/mL for amitriptyline and from 0.08 to 500 ng/mL for nortriptyline in rat plasma with correlation coefficients of >0.99. The lower limit of quantitation was 0.1 ng/mL for amitriptyline and 0.08 ng/mL for nortriptyline.

Precision and Accuracy. Intra- and inter-day precision and accuracy data are shown in Table 2. The accuracy [calculated as the percent error in the calculated mean concentration relative to the nominal concentrations (RE)] of amitriptyline analysis ranged from −10.1% to 2.0% with coefficients of variation (CVs) of 2.1% to 3.0% and 3.7% to 6.7% for intra- and inter-day precision, respectively. The accuracy of nortriptyline analysis ranged from −12.5% to 13.8%, with a CV of 2.6% to 6.7% and 3.3% to 8.7% for intra- and inter-day precision, respectively. These results indicate that this method has acceptable precision and accuracy.

Table 2. The accuracy and precision of intra- and inter-day assays (n = 5)

Intra-day	Nominal concentration (ng/mL)	Mean calculated concentration (ng/mL)	RSD (%)	RE (%)
Amitriptyline	0.5	0.47	2.6	−6.4
	10	10.12	3.0	1.2
	250	245.25	2.1	−1.9
Nortriptyline	0.5	0.58	6.7	13.8
	10	8.89	3.5	−12.5
	250	248.57	2.6	−0.5

Inter-day	Nominal concentration (ng/mL)	Mean calculated concentration (ng/mL)	RSD (%)	RE (%)
Amitriptyline	0.5	0.51	6.7	2.0
	10	9.08	6.2	−10.1
	250	245.29	3.7	−1.9
Nortriptyline	0.5	0.56	8.7	10.7
	10	10.49	5.0	4.7
	250	244.87	3.3	−2.1

Recovery and Stability. Percent recoveries of amitriptyline and nortriptyline are shown in Table 1. For all samples, including amitriptyline, nortriptyline, and the IS, neither matrix effects nor the percent loss exceeded ± 20%. Therefore, no significant matrix effects or interference from endogenous compounds occurred in rat plasma. A summary of

Table 3. Stabilities of amitriptyline, nortriptyline in rat plasma (n = 6)

Concentration (ng/mL)	Storage condition	Stability (%)	Stability (%)
Amitriptyline		0.5 ng/mL	250 ng/mL
Short term in plasma	Room temperature, for 6 h	98.21	109.87
Process (extracted sample)	4 °C, for 24 h	100.19	104.99
Freeze–thaw cycle in plasma	–70 °C, after the third cycle	92.82	97.26
Long term in plasma	–70 °C, for 20 days	93.62	101.10
Stock solution	4 °C, for 20 days	94.82	103.95
Nortriptyline		0.5 ng/mL	250 ng/mL
Short term in plasma	Room temperature, for 6 h	96.05	95.67
Process (extracted sample)	4 °C, for 24 h	94.76	103.22
Freeze-thaw cycle in plasma	–70 °C, after the third cycle	103.29	89.27
Long term in plasma	–70 °C, for 20 days	107.61	103.63
Stock solution	4 °C, for 20 days	101.13	97.61

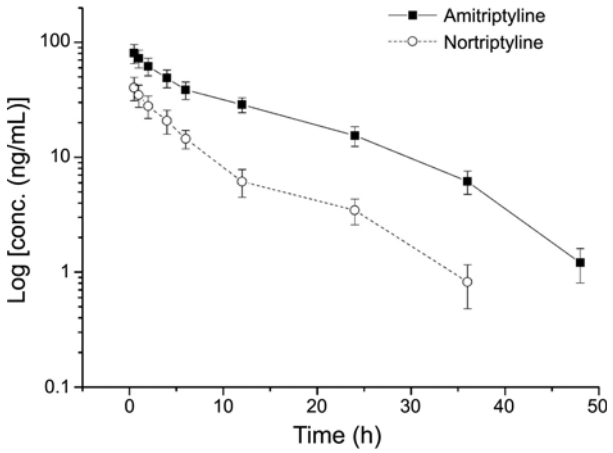

Figure 3. Mean plasma concentration-versus-time profile of amitriptyline and its metabolite (nortriptyline) in SD rats after oral administration of a single dose (15 mg/kg) of amitriptyline (n = 6).

Table 4. Pharmacokinetic parameters of amitriptyline, nortriptyline in rats after oral administration of 15 mg/kg amitriptyline (n =6)

PK parameters	Amitriptyline	Nortriptyline
C_{max} (ng/mL)	81.32 ± 3.74	40.11 ± 3.09
T_{max} (h)	0.35 ± 0.00	0.35 ± 0.00
AUC_{inf} (ng·h/mL)	992.47 ± 28.90	256.40 ± 28.56
$t_{1/2}$ (h)	8.27 ± 2.62	4.54 ± 0.87
Clearance/F (mL/h)	15.12 ± 0.43	N.A.

N.A.: not applicable

the assay stability under various conditions is presented in Table 3. The mean integrated peak areas of the LQC (lowest quality control) and HQC (highest quality control) samples were compared before and after the stability testing, as described in the Materials and Methods section. No stability issues were observed based on these experiments.

Method Application. The analytical procedures described above were employed to quantitate all analytes in plasma samples obtained from six SD rats that had been administered a single oral dose 15 mg/kg amitriptyline. The plasma concentrations of amitriptyline and nortriptyline are presented as a function of time in Figure 3. Model independent PK parameters are shown in Table 4. The C_{max} of amitriptyline was 81.32 ± 3.74 ng/mL at 0.35 h, the AUC_{inf} was 992.47 ± 28.90 ng·h/mL, and the half-life calculated from the terminal phase was 8.27 ± 2.62 h. The C_{max} of nortriptyline was 40.11 ± 3.09 ng/mL at 0.35 h, the AUC_{inf} was 256.40 ± 28.56 ng·h/mL, and the half-life calculated from the terminal phase was 4.54 ± 0.87 h.

Conclusions

A rapid, specific, and reliable LC-MS/MS-based bioanalytical method was successfully developed and validated to simultaneously determine amitriptyline and nortriptyline concentrations in rat plasma. This method applies simple LLE procedures, which reduces the preparation time and determines concentrations ranging from 0.1 to 500 ng/mL for amitriptyline and 0.08 to 500 ng/mL for nortriptyline using 0.2 mL of plasma. Our chromatographic conditions yielded shorter retention times (2.5-3 min) than any previous studies using LLE methods and accurately resolved peaks for both analytes. Moreover, the limit of quantitation concentration decreased to 0.1 ng/mL for amitriptyline. This is the lowest reported quantitation concentration for amitriptyline. The use of different collision energies can significantly lower background noise and interference peaks without reducing sensitivity. The assay also demonstrated a high degree of reproducibility and suitable precision and accuracy. This method allows laboratory scientists to rapidly determine amitriptyline and nortriptyline concentrations in plasma. The relatively short sample preparation time combined with the short LC runtime makes this method cost-effective and adaptable to high-throughput sample analysis.

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