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Simultaneous high-performance liquid chromatographic determination of caffeine and theophylline for routine drug monitoring in human plasma

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Abstract

An HPLC method for the simultaneous determination of both caffeine and theophylline in human plasma is described, using a reversed-phase chromatography column, heated by a thermostatic oven at 35°C, with UV detection and isocratic elution. The linearity and reproducibility of the method are verified. For the two drugs, the limit of detection is 0.1 μg ml^{-1}. This analytical method is rapid and reliable and allows routine controls of therapeutic levels of theophylline and caffeine, especially in premature infants where the volume of plasma samples is very small.

Keywords: Caffeine; Theophylline

1. Introduction

Theophylline has been widely used for the treatment of asthma and bronchospasm in adults. The efficiency and the toxicity of this drug may be modified by many factors [1]. Thus, in order to adapt dosing and to verify compliance, therapeutic drug monitoring is necessary. In order to treat neonatal apnea in children, caffeine is preferred to theophylline because of its better tolerance and its larger therapeutic range. According to the gestational age of the children, the kinetics of caffeine vary. This justifies therapeutic monitoring of caffeine in order to adapt dosing [2,3].

As summarized in Table 1, many HPLC procedures have been described for the determination of both caffeine and theophylline in different biological samples. Ultraviolet detection is widely used at 270–275 nm [1,3–10,18], 280 nm [2,11–15], 247 nm [16] or 230 nm [17], and also diode-array detection [17,18]. These methods often use C_{18}silica gel reversed-phase HPLC columns with isocratic elution with an acidic mobile phase. Gradient eluent has also been used [2,4,14,17,18]. β-Hydroxyethyltheophylline [1,3,11,14,18], 7-(β-hydroxypropyl)theophylline [4,13], 8-chlorotheophylline

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Table 1
Published HPLC procedures for the determination of caffeine and theophylline in biological samples

Biological samples and detected molecules	Sample volume (μl)	Preliminary extraction	Detection (nm)	Column	Mobile phase	pH	Flow-rate, (ml min^{-1}) elution gradient	Internal standard	Limit of detection	Ref.
Theophylline in plasma	100	Precolumn C_{18}	273	μBondapak C_{18} (300 × 3.9 mm I.D.), particle size 10 μm	Acetonitrile in sodium acetate buffer (80:920)	4	2.2	β-OH-ethyl-theophylline	0.5 μg ml^{-1}	[1]
Caffeine and metabolites in urine		Ammonium sulphate and chloroform–2-propranol (85:15)	280	μBondapak C_{18}	1.5–7.5% acetonitrile in 0.5% acetic acid		2 elution gradient			[2]
Caffeine in plasma	100	Chloroform in acidic pH (1 *M* HCl)	270	μBondapak C_{18} (250 × 4.6 mm I.D.)	Acetonitrile–water (1:9)		2	β-OH-ethyl-theophylline	0.2 μg ml^{-1}	[3]
Caffeine, theophylline theobromine and paraxanthine in breast milk		Protein precipitation with 6% perchloric acid	272	ODS C_{18} 250 × 4.6 mm I.D.), particle size 5 μm	0–16% methanol in 10 m*M* sodium acetate and 5 m*M* tetrabutyl-ammonium hydrogen-sulphate	4.9	1.5, elution gradient	7-(βOH-propyl) theophylline		[4]
Caffeine and metabolites in urine		Ammonium sulphate and chloroform–2-propranol (85:15)	275	Supelco C_{18}, 5 μm	15% Methanol in 0.05% acetic acid		1			[5]
Theophylline in serum (micellar liquid chromatography)	200	Precolumn C_{18} Corasil II (40 × 4 mm I.D.)	273	μBondapak phenyl (300 × 4 mm I.D.), particle size 10 μm	Micellar zwitterionic mobile phase 0.006 *M* 3-(dimethyldodecylammonio)-propanesulphonate–propanolol (97:3)			Antipyrine	0.5 μg ml^{-1}	[6]
Theophylline in serum	250	Bond Elut C_{18} cartridges eluted with methanol	272	Spherisorb C_{18} (220 × 4.6 mm I.D.), particle size, 5 μm	Methanol–0.038 *M* ammonium acetate–acetonitrile (38:57:5)	7.2		Caffeine		[7]
Theophylline in serum	40	Proteins precipitation with acetonitrile then extraction on Bond Elut C_{18} cartridges	272	LiChrosorb RP 18 ODS (250 × 4 mm I.D.) particle size 10 μm	Methanol 0.05 *M* ammonium acetate (42:58)	7	0.94	Theobromine	25 ng ml^{-1}	[8]
Theophylline, caffeine and anticonvulsants in plasma	50	Proteins precipitation with Zn sulphate	273	C_{18} (Waters) (150 × mm I.D.) size 5 μm	Acetonitrile–tetrahydrofuran–concentrated acetic acid–water (50:30:5:915)		1	Chlorotheophylline	5–6 μmol l^{-1}	[9]
Caffeine and metabolites in plasma	500	Dichloromethane in acidic pH (HCl)	274	TSK gel ODS-80 TM (150 × 4.6 mm I.D.), particle size 5 μm	Methanol–0.1 *M* sodium phosphate (30:70)		0.8	8-Chlorotheophylline	Caffeine and theobromine: 5 ng ml^{-1} Theophylline and paraxanthine: 10 ng, ml^{-1}	[10]

Caffeine in plasma	100	Bond Elut C_{18} cartridges eluted with acetone	280	C_{18}, particle size 5 μm	Acetic acid–acetonitrile–water (2:6:92)		1.5	β-OH–ethyltheophylline		[11]
Caffeine and theophylline in serum	5	Direct injection	280	Chrom-Spher 5 Bio-Matrix (150 × 4.6 mm I.D.)	Acetonitrile–water (50:950)		1	Theobromine		[12]
Theophylline in plasma + fluoroquinolones (ion-pair)		Chloroform–2-propanol or dichloromethane–2-propanol	280	Spherisorb S 5 ODS-2 (250 × 4 mm I.D.) + guard column	15% Acetonitrile in phosphate buffer containing 4.4 m*M* tetrabutylammonium hydrogensulphate	3	1.3	β-OH–etyyltheophylline		[13]
Caffeine and metabolites in urine and plasma	100 (urine)	Urine: 1 M NaOH, then 1 *M* HCl + mobile phase	200	Spherisorb ODS 2.5 μm (250 × 4.5 mm I.D.)	2–8% Acetonitrile in 0.5% acetic acid		Elution gradient	Urine: 4-acetaminophenol	0.2–1 μg ml^{-1}	[14]
	50 (plasma)	Plasma: perchloric acid, then 1 *M* NaOH, then extraction with chloroform at acidic pH (1 *M* HCl)	280	Chrom Sep (100 × 4.5 mm I.D.)	8 ml THF in 1000 ml 10 m*M* sodium acetate		1	Plasma: 4-aminophenol and β-OH-ethyltheophylline		
Caffeine and metabolites in urine	200 (urine)	Ammonium sulphate, chloroform–2-propanol (85:15)	280	ODS C_{18} (250 × 4.6 mm I.D.), 5 μm	0.05% Acetic acid–methanol (88:12)		1.2	N-Acetyl-*p*-aminophenol	0.3–0.5 μg ml^{-1}	[15]
Theophylline in serum and saliva	50 (plasma) 50 (saliva)	Protein precipitation with 20% perchloric acid	247	Hypersphere 3 C_{18} (100 × 4.6 mm I.D.) at 36°C and a guard column	Acetonitrile–methanol–phosphate buffer (4:7:89)	4,4	2	Sulphapyridine	25 ng ml^{-1} in serum, 50 ng mf^{-1} in saliva	[16]
Theophylline and several drugs in blood (post mortem)	250 (blood)	Protein precipitation with acetonitrile	Photodiode array at 230 and 214 nm and UV at 230 nm	Spherisorb 5S ODS 2 (150 × 3.8 mm I.D.) and a guard column	10–60% Acetonitrile in phosphate buffer	3.1	1, elution gradient	*p*-Methylphenylphenylhydantoin (MPPH)		[17]
Theophylline, caffeine and metabolites in plasma	100	Celute-MX sample preparation tubes. Elution with 2-propanol–dichloromethane (10:90)	Photodiode array at 285 and 273 nm and UV at 273 nm	ODS 3 μm (250 × 4.5 mm I.D.) at 50°C	0–25% Acetonitrile and 0.01–2% THF in acetate buffer	4	0.8, elution gradient	3-Ethylxanthine and β-OH-ethyltheophylline		[18]

[9,10] and theobromine [8,12] are the most frequently used internal standards.

An extraction procedure depending on the type of biological sample is generally used before performing liquid chromatography.

A few studies have indicated control of the column temperature [16–18]. This procedure may be criticized since the ambient temperature of the room in which the analysis is performed may vary throughout the year. Elsewhere, it has been reported that methylxanthines originating from dietary sources have an influence on the accuracy of caffeine and theophylline determination [19,20].

It was the objective of this investigation to develop a convenient, economic and rapid method for the determination of caffeine and theophylline in a small plasma volume.

2. Experimental

2.1. Chemicals

Anhydrous caffeine, theophylline and 7-(β-hydroxypropyl)theophylline were obtained from Sigma (St. Louis, MO, USA). Acetonitrile of HPLC grade and acetic acid were purchased from Carlo Erba (Milan, Italy), tetrahydrofuran of HPLC grade from Prochem (Wesel, Germany) and perchloric acid of analytical-reagent grade from Prolabo (Paris, France). Water was purified by using a Milli-Q Labo system (Millipore, Saint-Quentin, France).

A therapeutic drug monitoring serum (Lyphocheck, Bio-Rad, Paris, France) and a quality drug control serum (Ciba Corning Diagnostic, Irvine, CA, USA) were used.

2.2. Instrumentation

A Shimadzu analytical liquid chromatograph equipped with a Rheodyne Model 7125 syringe-load injector (50-μl loop), a programmable SPD 6 AV UV detector set at 273 nm (100% deflection equals 40 AUFS, absorbance range = 0.04), a Croco Cil heating system set at 35°C, a Chromatopac CR 6A recorder and an LC 6 A pump were used.

The analytical column was a stainless-steel reversed-phase ODS 2 column (150 × 4.6 mm I.D.) packed with Spherisorb C_{18}, 5 μm (Touzard-Matignon, Paris, France). The mobile phase used for HPLC separation was acetonitrile–tetrahydrofuran–concentrated acetic acid–distilled water (20:20:5:955). Elution was carried out at a flow-rate of 1 ml min^{-1} and at a column temperature of 35°C.

2.3. Standard solutions and internal standard solution

Stock standard solutions of caffeine and theophylline (2 mg ml^{-1}) were prepared in distilled water. Working standard solutions were prepared by dilutions of these stock standard solutions in order to obtain concentrations of 200, 500, 1000 and 1500 μg ml^{-1}. The internal standard was obtained by dissolving 20 mg of 7-(β-hydroxypropyl)theophylline in 100 ml of distilled water (stock standard solution), then diluted to 20 μg ml^{-1} (working standard solution). All these standards solutions were stable at 7°C for at least 3 months.

Caffeine- and theophylline-spiked blank plasma were used for calibration (concentration range 2–20 μg ml^{-1}). A 10-μl volume of each solution of caffeine and 10 μl of each solution of theophylline (200, 500, 1000, 1500 and 2000 μg ml^{-1}) were added to 980 μl of blank plasma in order to obtain final plasma concentrations of 2, 5, 10, 15 and 20 μg m^{-1}, respectively, for both caffeine and theophylline.

2.4. Extraction procedure

Blood samples were collected in heparinized glass tubes and centrifuged for 5 min at 2000 *g*. A 50-μl volume of the internal standard working standard solution (20 μg ml^{-1}) was added to 50 μl of plasma or blank plasma spiked with a known amount of working standard solutions. The sample was vortex mixed for 10 s, then 20 μl of 20% perchloric acid were added and the sample was vortex mixed for 10 s and centrifuged

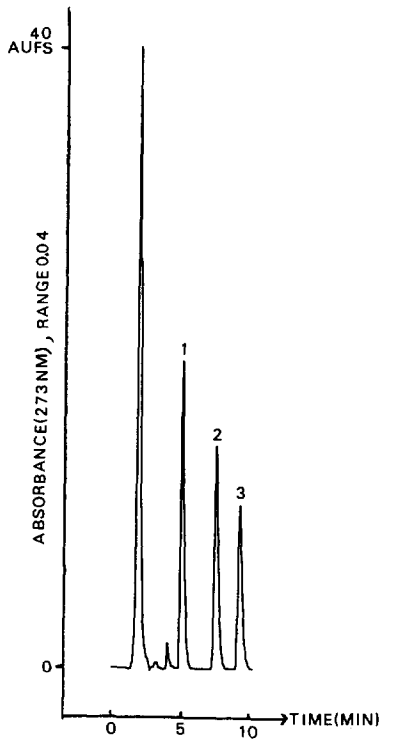

Fig. 1. Chromatogram of blank plasma spiked with caffeine and theophylline (20 μg ml^{-1} each) and with internal standard added. Peaks: 1 = theophylline; 2 = caffeine; 3 = internal standard.

at 2000 *g* for 5 min. The supernatant was then directly applied to the HPLC column. Under these conditions, the retention times of theophylline, caffeine and the internal standard were 5.0, 7.5 and 9.2 min, respectively (Fig. 1).

3. Results and discussion

3.1. Choice of the internal standard

As mentioned previously, several substances having a maxima in the UV spectra at about 273 nm were evaluated as possible internal standards, such as theobromine, 8-chlorotheophylline, β-hydroxyethyltheophylline and 7-(β-hydroxypropyl)theophylline. 8-Chlorotheophylline was not chosen because of the rapid degradation of the aqueous-stock standard solution (within 10 days this compound is changed into theophylline), theobromine was not chosen because its retention time was too short and occurred at the same time as the solvent front and under our experimental conditions, β-hydroxyethyltheophylline was not sufficiently separated from theophylline. Therefore, 7-(β-hydroxypropyl)theophylline was chosen as the internal standard, as its peak was sufficiently separated from that of caffeine and it was stable in an aqueous stock standard solution for more than 6 months (Fig. 2).

3.2. Validation procedure

Linearity

The responses of both theophylline and caffeine varied linearly with concentration between 2 and 40 μg ml^{-1}. The two drugs had a correla-

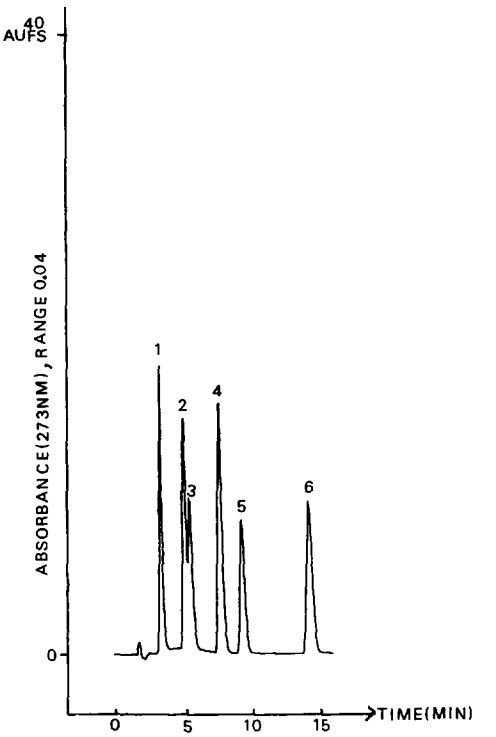

Fig. 2. Chromatogram of an aqueous mixture of (1) theobromine (10 μg ml^{-1}), (2) theophylline (10 μg ml^{-1}), (3) β-hydroxyethyltheophylline (5 μg ml^{-1}), (4) caffeine (15 μg ml^{-1}), (5) 7-(β-hydroxypropyl)theophylline (5 μg ml^{-1}) and (6) 8-chlorotheophylline (5 μg ml^{-1}).

tion coefficient of 1.00. The regression curves were $y = 0.062x - 0.002$ and $y = 0.089x + 0.006$ for caffeine and theophylline, respectively.

Reproducibility

The within-day and between-day precisions of the method were investigated by using several aliquots of a blank plasma sample spiked with caffeine and theophylline at concentrations of 5, 10 and 20 $\mu g\ ml^{-1}$. Concerning the within-day precision, ten samples were analysed for caffeine and theophylline on the same day. Concerning the between-day precision, ten measurements were made over 1 month. The results are given in Table 2.

Recovery

The recoveries of caffeine and theophylline were determined using a blank plasma sample spiked with increasing concentrations of the two drugs, i.e., 2, 5, 10 and 20 $\mu g\ ml^{-1}$; the absolute recovery was expressed as the percentage of the directly injected standard solutions of the drugs. As shown in Table 3, the recovery varied from 97 to 99% and from 98 to 101% for caffeine and theophylline, respectively.

Detection limits

The limit of detection was 0.1 $\mu g\ ml^{-1}$ for both caffeine and theophylline at a signal-to-noise ratio of ca. 3:1.

Table 3
Recoveries in blank plasma with increasing and known amounts of caffeine and theophylline added

Added amount ($\mu g\ ml^{-1}$)	Caffeine		Theophylline	
	Recovered ($\mu g\ ml^{-1}$)	Recovery (%)	Recovered ($\mu g\ ml^{-1}$)	Recovery (%)
2	1.98	99	1.98	99
5	4.85	97	4.90	98
10	9.80	98	10.0	100
20	19.80	99	20.2	101

Interferences

Endogenous plasma components did not interfere with either caffeine or theophylline. The blank plasma (Fig. 3) used for calibration and validation procedure studies and endogenous components from dietary sources [19,20] were tested in order to determine the absence of caffeine and theophylline. By using quality controls and serum from Bio-Rad as an internal control, we evaluated the possibility of technical interferences produced by some commonly used drugs such as analgesics, antibiotics and anticonvulsants, as shown in Table 4. These drugs did not interfere. Further, some substances evaluated as internal standards such as theobromine, 8-chlorotheophylline and β-hydroxyethyltheophylline were found not to interfere with caffeine and theophylline (Fig. 2).

Table 2
Precision of the method[a]

Concentration ($\mu g\ ml^{-1}$)	Compound	Within-day reproducibility			Between-day reproducibility		
		Mean ($\mu g\ ml^{-1}$)	S.D. ($\mu g\ ml^{-1}$)	C.V. (%)	Mean ($\mu g\ ml^{-1}$)	S.D. ($\mu g\ ml^{-1}$)	C.V. (%)
5	Theophylline	4.91	0.114	2.30	4.95	0.168	3.43
	Caffeine	4.86	0.121	2.45	4.95	0.157	3.23
10	Theophylline	10.05	0.171	1.70	9.98	0.238	2.25
	Caffeine	10.08	0.131	1.38	9.86	0.186	1.89
20	Theophylline	20.28	0.371	1.83	20.12	0.369	1.84
	Caffeine	19.88	0.388	1.95	19.93	0.513	2.57

[a] Within-day reproducibility: ten samples were analysed on the same day (blank plasma sample spiked with caffeine and theophylline at concentrations of 5, 10 and 20 $\mu g\ ml^{-1}$). Between-day reproducibility: ten measurements were made with the same pools of plasmas during 1 month.

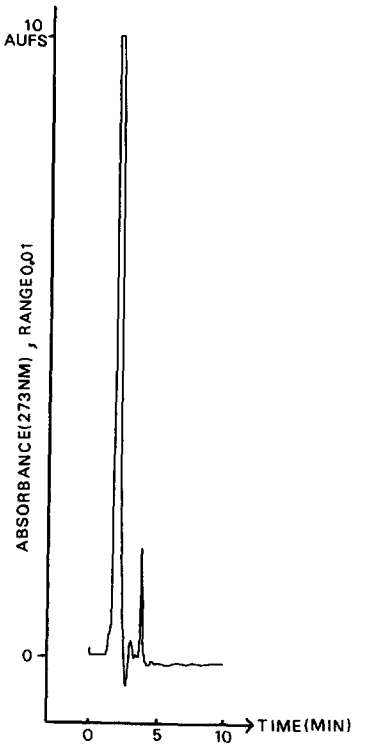

Fig. 3. Chromatogram of a blank plasma under conditions suitable for the analysis of samples at the lowest limit of the calibration range (100% deflection equals 10 AUFS).

Table 4
Selectivity[a]

Sample	Compound	Level 1	Level 2
Quality control type I	Carbamazepine	5.7	13.6
	Phenytoin	5.2	21.2
	Phenobarbital	11.0	32.0
Quality control type II	Quinidine	1.9	5.2
Bio-Rad	Acetaminophen		38.0
	Amitriptyline		0.174
	Carbamazepine		8.5
	Chloramphenicol		15.7
	Desipramine		0.136
	Disopyramide		4.1
	Ethosuximide		72.0
	Imipramine		0.139
	Lidocaine		4.0
	NAPA		5.0
	Nortriptlyine		0.098
	Phenobarbital		22.0
	Phenytoin		13.0
	Primidone		4.5
	Procainamide		10.2
	Propranolol		0.082
	Quinidine		3.6

[a] Concentrations (μg ml^{-1}) in Bio-Rad serum and quality controls which did not interfere with caffeine and theophylline determination.

3.3. Comparisons with other techniques

Compared with the previous methods used in our laboratory for drug monitoring of caffeine (Syva-Biomerieux) and theophylline (Abbott), such as immunoenzymatic assays (Syva-Biomerieux), the present method showed good correlations: on the same set of patients' samples, the correlation coefficient was $r = 0.956$, $p = 0.0001$ and $r = 0.819$, $p = 0.0001$ for theophylline and caffeine, respectively. However, the present method is less expensive and did not show any interferences.

Compared with the other HPLC techniques summarized in Table 1, the present method has several advantages:

(i) Only a small volume of plasma sample (50 μl) is needed, which is of particular interest for the determination of caffeine in premature infants. A few studies using such a volume [8,9,12,16] have been described, but they did not assay both theophylline and caffeine or apply control of the column temperature [9,12].

(ii) The extraction procedure is limited to a deproteinization with perchloric acid, which allows rapid processing.

(iii) The control of the column temperature, set at a constant value of 35°C, explains the particular stability of the retention times, which is appreciated in a daily routine technique. During a period of 2 months, the variation of retention times was less than 0.5% (intra-assay) and 2% (inter-assay).

This method has been used in our laboratory for 2 years for drug monitoring of both caffeine and theophylline, especially in order to adapt caffeine dosing in premature infants. Examples of chromatograms are given in Fig. 4. As mentioned previously, no drug interference was

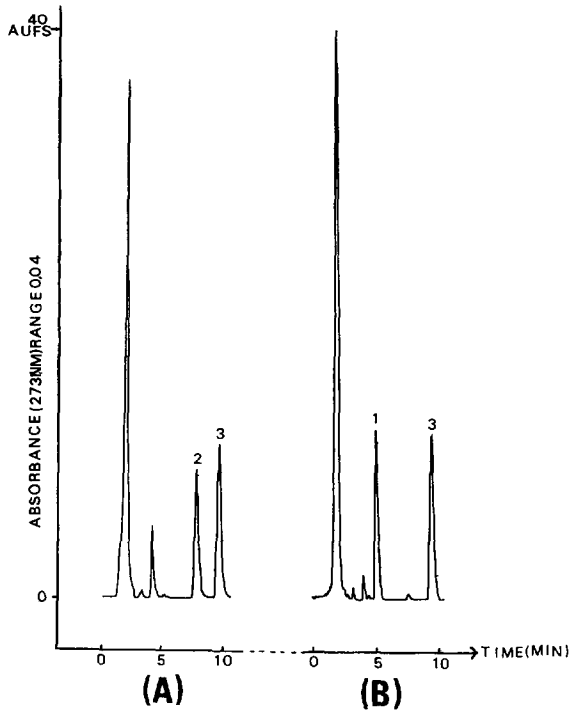

Fig. 4. Chromatograms of patients' plasma, with internal standard added. (A) Premature infant treated with caffeine (12.2 μg ml^{-1}); (B) adult treated with theophylline (10.6 μg ml^{-1}). Peaks: 1 = theophylline; 2 = caffeine; 3 = internal standard.

found. In our daily practice, the Bio-Rad internal control serum (level 2) is applied every day and quality controls of these two drugs are performed twice a week. All the controls were in the correct range. Taking into account the stability of the aqueous solutions, a calibration graph is obtained every 6 weeks. The column may be changed every 6 months, which, in our daily routine, corresponds to ca. 1000 injections.

4. Conclusion

The described technique for caffeine and theophylline drug monitoring is very rapid, easy to carry out, not very expensive, simple, specific, accurate and reliable. This technique is suitable for daily routine drug monitoring, especially in premature children where the volume of plasma samples is usually very small.

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