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Monitoring of tricyclic antidepressants in human serum and plasma by HPLC: characterization of a simple, laboratory developed method via external quality assessment

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Abstract

A reversed-phase high performance liquid chromatography (HPLC) method for the determination of plasma and serum levels of amitriptyline (AMI), nortriptyline (NORT), imipramine (IMI), desipramine (DESI), clomipramine (CLOMI), and norclomipramine (NCLOMI) is described. The assay is based upon single step liquid/liquid extraction of these compounds using hexane at pH 11 (recovery between 92 and 105%), a Nova-Pack C-18 HPLC cartridge column, a mobile phase composed of a phosphate buffer with 50% (v/v) acetonitrile and about 0.2% (v/v) diethylamine (final pH: 8) and solute detection at 242 nm. Using 1 ml of plasma or serum and econazole as internal standard, drug levels between 20 and 400 ng ml^{-1} (about 60–1450 nM) were found to provide linear calibration graphs. For drug concentrations in the range of 70–120 ng ml^{-1} (about 240–430 nM), intraday and interday imprecisions ($n = 5$) were determined to be < 6.0, and $< 15\%$, respectively. Data reported include those gathered over a 3-year period during which this assay was employed for therapeutic drug monitoring and clinical toxicology. The performance of the laboratory developed assay was assessed via analysis of monthly samples provided by an external quality control scheme.

Keywords: Tricyclic antidepressants; Reversed-phase liquid chromatography; External quality assessment; Therapeutic drug monitoring; Clinical toxicology

1. Introduction

Tricyclic antidepressants (TCA) are commonly used for the treatment of depressive disorders and many studies have indicated that pharmacotherapy with these compounds should be accompanied by therapeutic drug monitoring (TDM). Furthermore, TCAs are frequently encountered in clinical and forensic samples that are associated with drug overdose. Thus, monitoring of TCAs in body fluids, including serum and plasma, became a must for many major drug assay laboratories [1,2]. Reported methods for analysis of TCAs in

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serum and plasma include immunological techniques and chromatographic approaches. Immunoassays, including those based upon fluorescence polarization immunoassay (FPIA, [3,4]), and enzyme multiplied immunoassay technique (EMIT, [5]), may be utilised to rapidly screen patient samples for these drugs as a group. They are therefore mainly employed to detect toxic concentrations of TCAs. Without elaborate sample pretreatments, levels of single TCAs in presence of metabolites of similar structure can thereby not be assessed [5,6]. Chromatographic assays, on the other hand, provide data for single compounds, i.e. they permit the identification of the administered drugs and their major metabolites. In the past 20 years, many instrumental approaches based upon gas chromatography (GC) and high performance liquid chromatography (HPLC) have been developed and applied to routine application [2,7–10]. Recent contributions to the HPLC literature mainly focus on alternate or improved extraction and detection schemes [11–13]. Alternatively, one paper reports the determination of TCAs in human plasma using micellar electrokinetic capillary chromatography [14].

A few years ago, our laboratory was asked to provide service for selective analysis of five commonly prescribed TCAs, namely amitriptyline (AMI), nortriptyline (NORT), imipramine (IMI), desipramine (DESI) and clomipramine (CLOMI). Furthermore, serum levels of a major metabolite of CLOMI, norclomipramine (NCLOMI), were also requested. Although these drugs are not administrated together for pharmacotherapeutic reasons, our goal was to have a single and simple HPLC method for all these substances that could be employed for TDM and clinical toxicology. As no simple commercial HPLC assay was available for these six compounds, an in-house reversed-phase HPLC method was developed. Emphasis was directed toward an approach with a single step liquid/liquid extraction procedure and the use of commercially available sample vials without silanisation of glass walls. In this paper, the laboratory developed assay is described and its performance is assessed with the data of external quality control samples that were analyzed over a 3-year period. To our knowledge, no such assay characterization has previously been reported in the literature describing the analysis of TCAs in blood. Furthermore, the use of the HPLC assay to provide insight into clinical toxicology specimens is demonstrated.

2. Materials and methods

2.1. Drugs, chemicals, origin of patient sera, external quality control samples and bovine plasma

The six tricyclic antidepressants, all as hydrochlorides, were received from Sigma (Basel, Switzerland). Econazole was obtained from Cilag Chemie (Schaffhausen, Switzerland). Methanol and acetonitrile of HPLC grade were from Biosolve (Amsterdam, The Netherlands), hexane was from Merck (Darmstadt, Germany), diethylamine was from Fluka (Buchs, Switzerland) and all other chemicals were of analytical grade. Patient sera were collected in the departmental routine drug assay laboratory where they were received for therapeutic drug monitoring and clinical toxicology. Heathcontrol EQA external quality control samples were purchased from Cardiff Bioanalytical Services (Cardiff, UK) as lyophilised human serum and were reconstituted in 5 ml water. Bovine plasma was received from a local slaughter house. All samples were stored at −20°C in polypropylene vials.

2.2. Standard solutions, calibration and control samples

Stock solutions of all six constituents were prepared with methanol (200 μg ml^{-1} of each hydrochloride) and stored at −20°C. For daily use, 30 μl aliquots of these solutions were combined and diluted to 3 ml with methanol. Aliquots of this mixture were added to bovine plasma providing drug levels of 20, 50, 100, 150, 200, 300 and 400 ng ml^{-1} (about 60–1450 nM, conversion factors for AMI, NORT, IMI, DESI, CLOMI, and NCLOMI, being 3.61, 3.80, 3.57, 3.75, 3.18, and 3.34, respectively). Control samples contain-

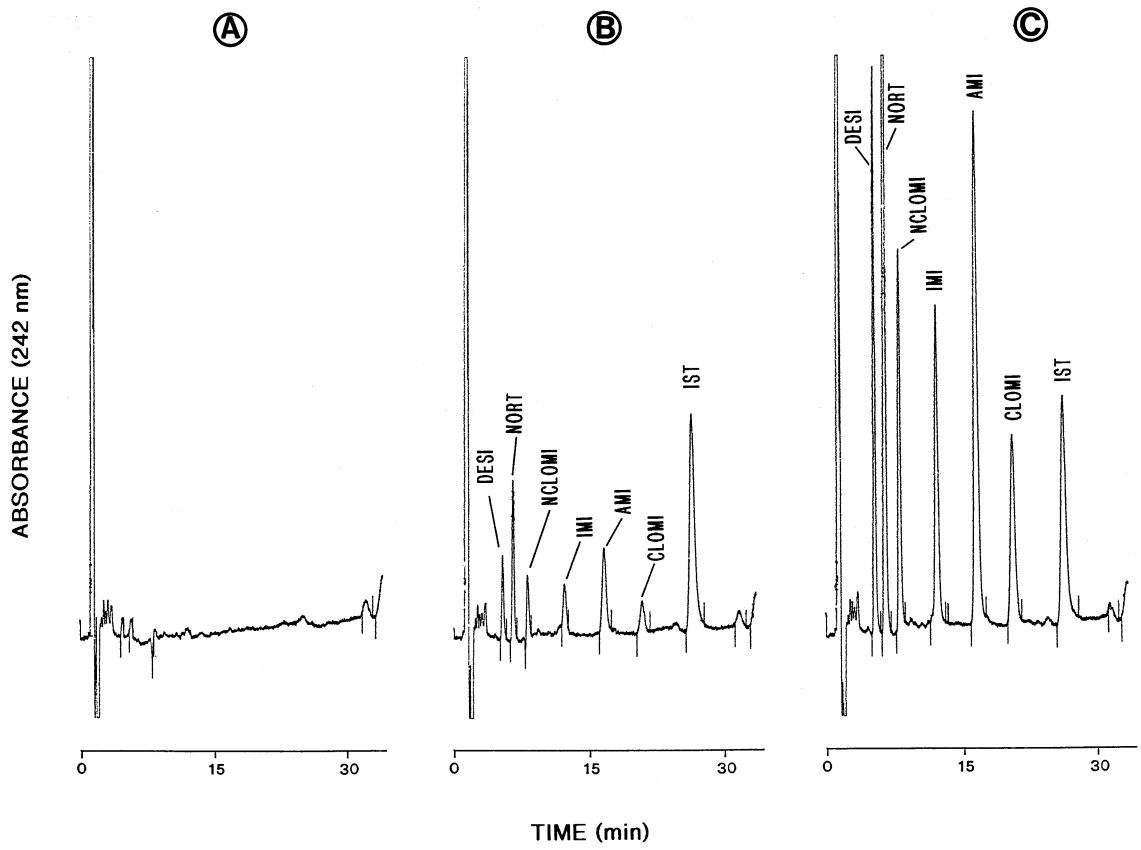

Fig. 1. Typical chromatograms of (A) a blank sample, (B) a calibrator containing 50 ng ml^{-1} of each TCA, and (C) a calibrator with 300 ng ml^{-1} of each TCA.

ing drug levels in the range of 75–120 ng ml^{-1} (240–430 nM) were prepared in the same way.

2.3. Extraction

A total of 1 ml of patient, calibrator or control serum/plasma was combined with 50 μl of internal standard solution (methanolic solution of econazole, 90 μg ml^{-1}), 1 ml of 0.1 M sodium tetraborate solution (adjusted to pH 11 with 30% NaOH) and 6 ml hexane in a 10 ml Sovirel glass tube that was rinsed carefully with methanol. After gently shaking the closed tube for 10 min and centrifugation at 3000 rpm for 10 min, the hexane (upper) phase was transferred to a clean test tube and evaporated to dryness at 40°C employing a gentle stream of air. The residue was redissolved in 200 μl of methanol.

2.4. HPLC conditions

HPLC analyses were performed using a model 510 pump (Waters Associated, Milford, MA, USA), a model 717 plus autosampler (Waters), a reversed-phase C18 column (Nova-Pak C18 60A 4 μm, 4.6 × 150 mm, Waters) and a model Spectraflow 757 UV detector (Kratos Analytical, Ramsey, NJ, USA). The mobile phase was prepared by mixing 500 ml of 5 mM aqueous KH_2PO_4 buffer, 500 ml acetonitrile and 2 ml diethylamine and by adjusting the pH to 8 by addition of concentrated phosphoric acid. The flow rate was 0.9 ml min^{-1} (pressure about 2100 psi), the temperature was ambient and detection was effected at 242 nm. A total of 35 μl of the methanolic extract were injected (from a glass vial) and the run time was 34 min. A model HP

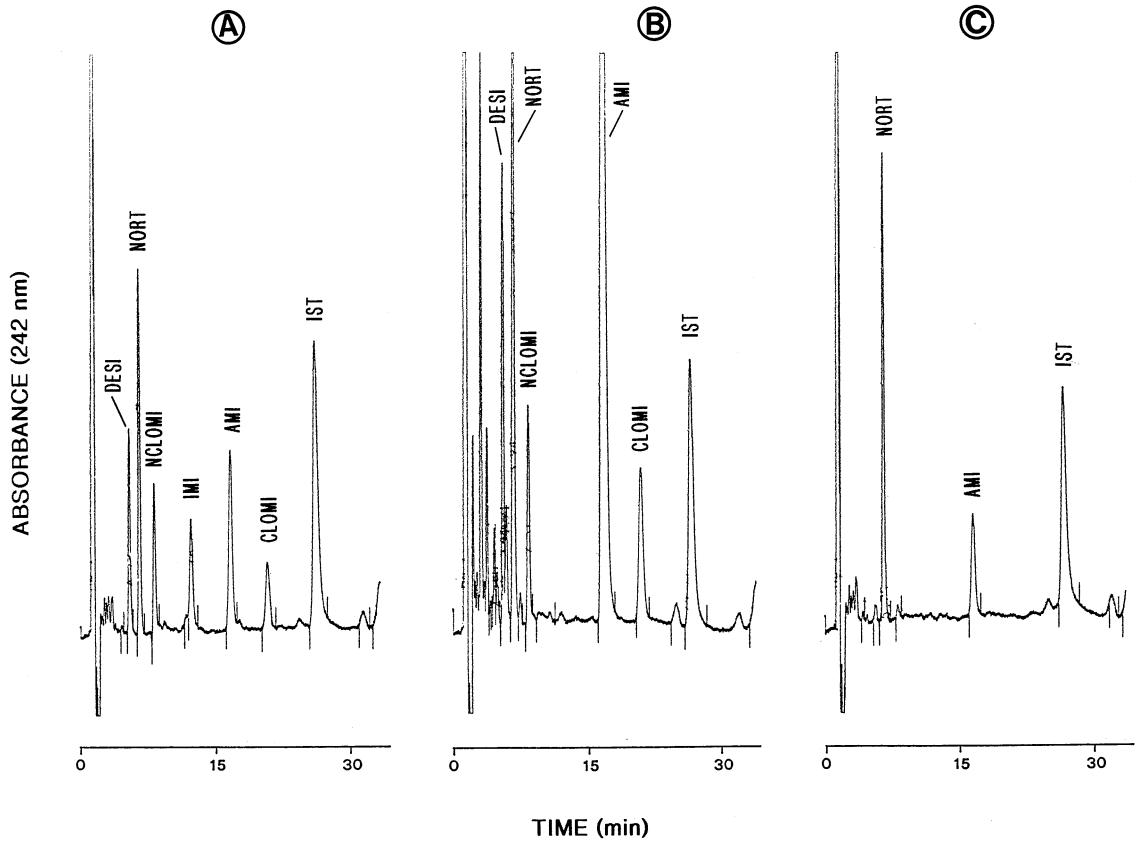

Fig. 2. Chromatograms of (A) a control sample prepared in bovine plasma, (B) a sample of a patient with suspected TCA overdose, and (C) an external quality control sample.

3396 Series III integrator (Hewlett Packard, Widen, Switzerland) was used for data registration. Quantitation was based upon internal, seven-level calibration using peak areas.

2.5. FPIA

Patient samples with suspected TCA intoxication were also analyzed by an automated fluorescence polarization immunoassay (FPIA) method on the TDxFLx Analyzer (Abbott Laboratories, Irving, Texas, USA). The FPIA assay was performed according to the manufacturers instructions. This assay employs IMI as calibrating substance in the range between 0 and 1000 ng ml^{-1} (0–3570 nM) and has a detection limit for IMI of 20 ng ml^{-1} (71.4 nM). For a drug level of 500 ng ml^{-1}, crossreactivities for DESI, AMI, NORT, CLOMI, and NCLOMI, are reported to be 105, 109, 81, 41, and 61%, respectively, [15]. Internal quality control assessed over one year revealed interday imprecisions of 7.56, 6.49 and 4.53% for drug levels of 100 ng ml^{-1} (mean: 96.4 ng ml^{-1}; $n = 29$), 200 ng ml^{-1} (mean: 202.2 ng ml^{-1}; $n = 36$), and 500 ng ml^{-1} (mean: 497.4 ng ml^{-1}; $n = 43$), respectively.

3. Results and discussion

3.1. Description of the assay

Data obtained with calibration samples are depicted in Fig. 1. For the blank bovine plasma extract (panel A of Fig. 1), no major peak was detected. Typical chromatograms obtained with

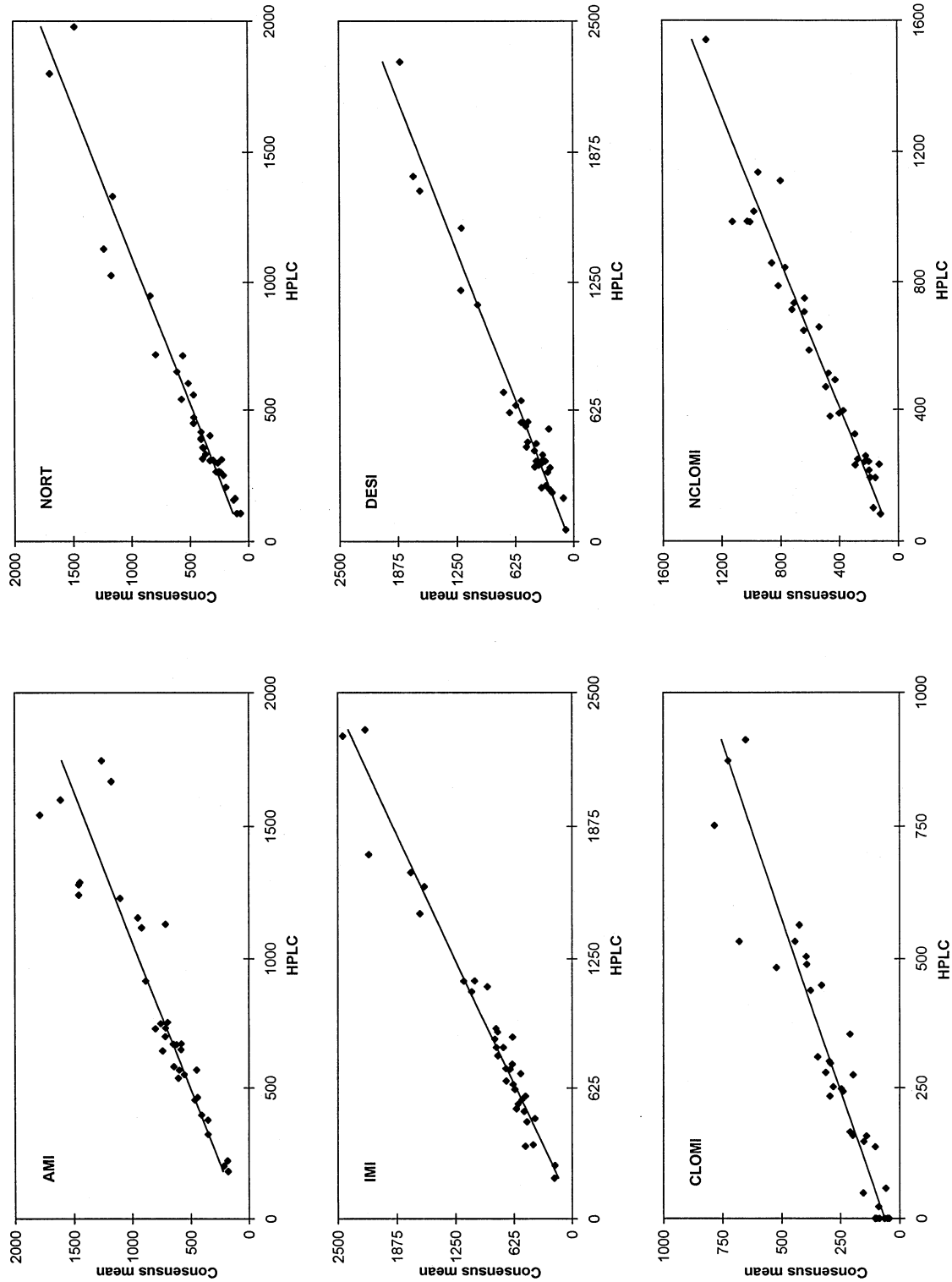

Fig. 3. Comparative drug levels of external quality control samples (our HPLC data versus consensus mean values). The units are nM in all cases.

Table 1
Typical intraday and interday imprecision data

Drug	Intraday (same day)[a]			Interday (different days)[a]		
	Drug level (nM)	Mean (nM)	RSD (%)	Drug level (nM)	Mean (nM)	RSD(%)
AMI	337	303	3.58	268	292	3.50
NORT	282	275	3.91	275	296	5.77
IMI	385	379	2.58	262	279	12.7
DESI	423	408	5.48	270	278	13.2
CLOMI	316	346	4.80	242	259	12.8
NCLOMI	293	284	1.78	275	263	14.2

[a] $n = 5$.

blank bovine plasma that was spiked with 50 and 300 ng ml^{-1} of each of the six TCAs and the IST (econazole, 4.5 μg ml^{-1} plasma), are depicted in panels B, and C, of Fig. 1, respectively. The TCAs and the IST are shown to be well recovered and separated. The recovery was determined by comparing peak areas after extraction with peak areas obtained by direct injection of equal amounts of the drugs. Employing mean values of triplicates and drug levels around 600 nM, recoveries of AMI, NORT, IMI, DESI, CLOMI and NCLOMI were found to be 99.0, 92.4, 101.8, 91.6, 104.8, and 92.1%, respectively. After evaluation of C8, C18 and C_6H_5-HPLC columns of various manufacturers, the Nova-Pak C18 column was found to provide the best separation of the six TCAs. Prolonged use of the column resulted in somewhat increased retention of the TCAs but not of the IST. This tendency could be counterbalanced by reducing the pH of the mobile phase. Thus, if required (elimination of interference of CLOMI with IST), the pH of the mobile phase was lowered by one or two tenths of a pH unit. Furthermore, it is worth mentioning that new columns had to be equilibrated carefully by injecting about 15 times the methanolic solution containing the IST. Without this procedure, a strongly tailing IST peak was monitored. Typical chromatograms obtained with extracts of a control, a patient and an external quality control sample are depicted in Fig. 2.

Quantitation of TCAs was based upon internal, seven-level calibration using the peak area ratio of the compound to the IST and having a 20–400 ng ml^{-1} (about 60–1450 nM, see above) concentration range. During the course of this work, more than 80 calibrations were undertaken. All calibration graphs were determined to be linear with F values > 250 ($P < 0.001$), the regression coefficients r being > 0.990. The y-intercepts were observed to be significantly smaller than the smallest calibrator values and were thus negligible. Having control samples with drug levels between 240 and 430 nM, intraday and interday imprecision expressed via relative standard deviations (RSD) were determined to be $< 6.0\%$ ($n = 5$), and 15% ($n = 5$), respectively, Table 1. The data presented in Fig. 2(A) correspond to a control sample that comprised about 75 ng ml^{-1} of each TCA. With the assay conditions described in Section 2, the detection limit (S/N = 3) was found to be about 60 nM (range: 57–67 nM) for all six compounds of interest. This sensitivity is sufficient for the determination of the TCA serum/plasma levels in clinical samples.

3.2. Assay characterization via external quality assessment

The data presented in Fig. 2(C) represent those obtained with an external quality control sample that contained AMI and NORT. The concentrations determined by HPLC, 218 and 560 nM, respectively, were found to compare well with the reported spiking values of 208 and 553 nM, respectively. Similarly, 3-monthly samples containing AMI/NORT, IMI/DESI, and CLOMI/NCLOMI, respectively, were analyzed

Table 2
Statistical, linear regression analysis and bias analysis data of comparative drug levels in 35 external quality control samples

Drug	HPLC data		Reported data of quality assurance scheme[a]			Linear regression analysis data[b]			Bias analysis data[c]	
	Mean (nM)	Median (nM)		Mean (nM)	Median (nM)	Slope	y-Intercept (nM)	r	Mean (nM)	SD (nM)
AMI	807.9	670	Spiked value	822.5	699	0.959	47.9	0.923	14.6	178.2
			Consensus mean	774.4	699	0.879	64.5	0.924	−33.4	165.6
			RP HPLC mean	776.3	699	0.861	71.2	0.924	−31.6	168.7
NORT	538.7	386	Spiked value	539.4	428	0.926	40.4	0.980	0.743	89.17
			Consensus mean	510.1	392	0.869	42.0	0.975	−28.6	106.0
			RP HPLC mean	525.0	408	0.909	34.2	0.978	−13.7	94.28
IMI	889.5	740	Spiked value	946.4	740	1.113	−43.8	0.976	56.9	142.7
			Consensus mean	881.7	709	1.051	−53.0	0.978	−7.83	118.3
			RP HPLC mean	891.8	737	1.053	−48.3	0.977	2.31	124.1
DESI	622.3	448	Spiked value	629.3	504	0.861	93.8	0.971	7.06	127.9
			Consensus mean	576.1	418	0.864	38.5	0.981	−46.1	110.2
			RP HPLC mean	584.9	413	0.896	25.9	0.982	−37.4	100.2
CLOMI	291.8	252	Spiked value	301.4	249	0.807	65.8	0.926	9.57	94.60
			Consensus mean	285.6	246	0.755	65.2	0.944	−6.20	89.46
			RP HPLC mean	291.3	242	0.785	61.9	0.950	−0.571	83.32
NCLOMI	578.3	514	Spiked value	574.0	511	0.909	47.5	0.952	5.09	108.5
			Consensus mean	549.9	491	0.883	39.2	0.971	−28.4	88.29
			RP HPLC mean	556.1	486	0.917	24.7	0.971	−22.2	86.21

[a] The consensus mean represents the mean of all data reported by 30–40 laboratories (independent of analytical method used); RP HPLC mean is the mean of reversed-phase HPLC data reported by 10–20 laboratories.

[b] HPLC data and reported data were taken as x-axis, and y-axis, respectively.

[c] To analyze the bias, the mean and SD of the difference of each data pair (reported value-HPLC value) was calculated.

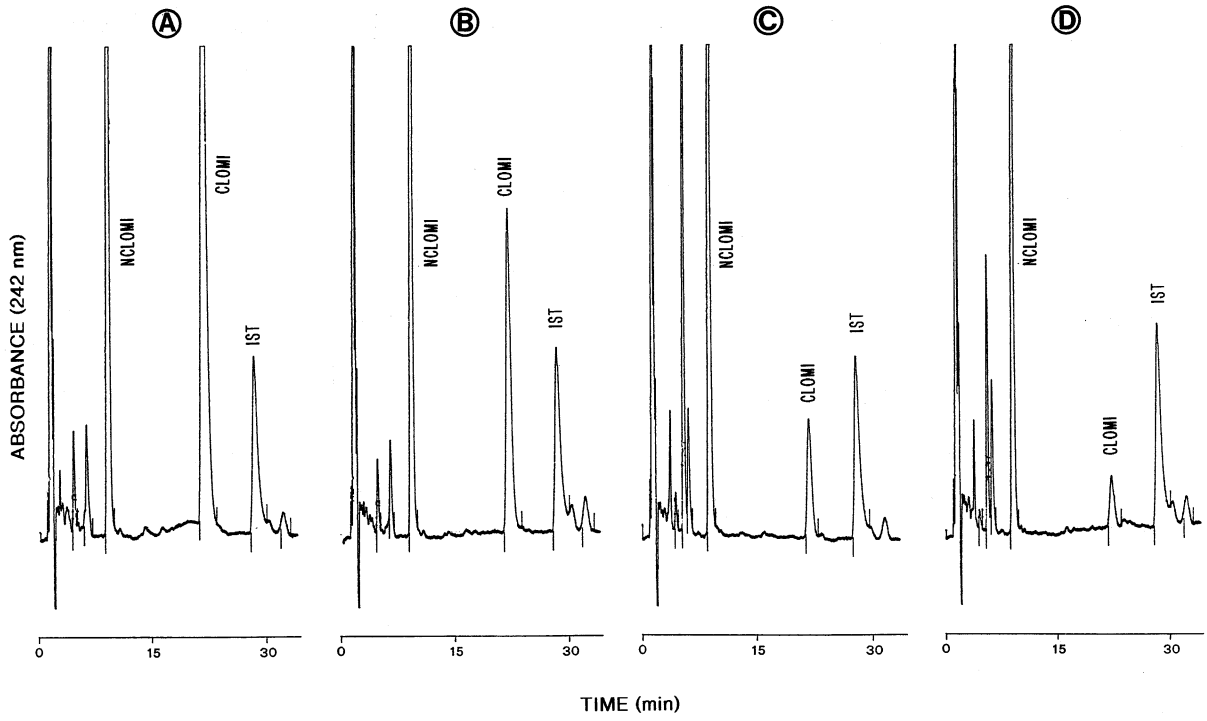

Fig. 4. Chromatograms of sera depicting a CLOMI intoxication (A) at the time of the patient's admission to the hospital, (B) 1 day, (C) 4 days, and (D) 6 days thereafter.

over a 3-year period and the data obtained were sent to the organisers of the external quality assurance scheme. Retrospectively, the data of 35 months (one sample set was not analyzed) could be compared and the results are summarised in Table 2. Our HPLC data were compared with the reported spiking values, with the mean of the data obtained in other laboratories (denoted as consensus mean, data that were monitored with different techniques) and with the mean of the values obtained in other laboratories using assays that are based upon reversed-phase HPLC (denoted as RP HPLC mean). For all six compounds, mean and median values were found to be very similar. Statistical analysis using the Mann–Whitney rank sum test revealed the absence of a statistically significant difference between each pair of input groups ($P > 0.6$). Furthermore, linear regression analysis of comparative data pairs revealed linear relationships with relatively small y-intercepts and slopes that were reasonably close to unity (Table 2). One set of graphs is presented in Fig. 3. The three graphs obtained for each drug were found to be very similar (Table 2). Strongest discrepancies between our data and those obtained in other laboratories (and the spiking levels, data not shown), were noted for higher concentrations of AMI and CLOMI. As our data are based upon single wavelength solute detection, possible interferences in our chromatoograms cannot be excluded without data gathering at multiple wavelengths. Furthermore, the bias expressed by the mean of the differences of the data pairs was found to be small, particularly in relation to the concentration range monitored.

3.3. TDM and clinical toxicology

The described HPLC assay was used to provide TDM and clinical toxicology data for the local university hospital. The chromatogram depicted in Fig. 2(B) resulted from an analysis of the serum

of a patient with suspected TCA intoxication who was admitted to the emergency care unit. FPIA analysis of this sample revealed the presence of a high amount of TCAs. The response obtained corresponded to that of 5008 nM of IMI. Although this result manifested the intoxication, it did not reveal the compounds involved. HPLC analysis provided the presence of five TCAs, namely AMI (2484 nM), NORT (798 nM), DESI (668 nM), CLOMI (556 nM) and NCLOMI (428 nM). No IMI was detected, suggesting that the patient was taking AMI (NORT is the metabolite of AMI), DESI and CLOMI (NCLOMI is the metabolite of CLOMI). The concentrations monitored are all above the established therapeutic levels [1]. Furthermore, it was interesting to find that the sum of the concentrations monitored in that sample (4934 nM) was very close to the value obtained by FPIA. Moreover, taking the reactivities between the drugs and the antibody into account (data at 500 ng ml^{-1} concentrations, see above), a total value of 4537 nM was obtained. This comparison suggests that our HPLC assay monitored most of the molecules that contributed to the FPIA response.

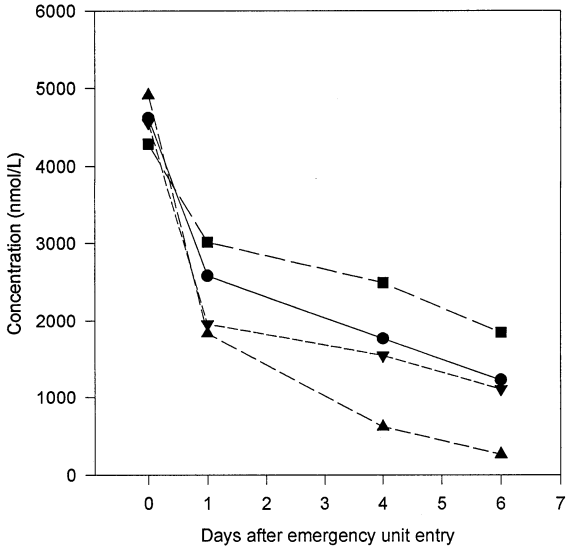

Fig. 5. TCA data of a hospitalised patient with CLOMI overdose (for chromatograms see Fig. 4). (▲) CLOMI and (■) NCLOMI serum levels assessed by HPLC, (▼) serum levels monitored by FPIA, and (●) serum levels calculated from the HPLC data and the FPIA crossreactivities for CLOMI and NCLOMI.

The data presented in Fig. 4 represent another example of a TCA intoxication. Although the FPIA response revealed a high amount of TCAs (Fig. 5), only HPLC data showed that the patient was heavily intoxicated with CLOMI and its metabolite NCLOMI. Using HPLC, the concentrations of the two compounds at the time of the patient's admission to the emergency care unit were determined to be 4908 and 4281 nM, respectively. Furthermore, generation of the specific HPLC data over several days after hospitalization of the patient provided insight into the elimination of the parent drug and its major metabolite (Fig. 5). Not surprisingly, the CLOMI serum level was found to decrease much faster compared to that of NCLOMI. Serum concentrations of NCLOMI and CLOMI were determined to be 1845 and 260 nM, respectively, 6 days after admission of the patient to the hospital (and without further administration of the drug). The data presented in Fig. 5 also contain drug levels that were calculated from the HPLC data and the FPIA crossreactivities for CLOMI (41%) and NCLOMI (61%). Except for the value at 1 day after hospitalization, these data were found to compare well with those obtained by FPIA.

4. Conclusions

In this paper, the performance of a simple, laboratory developed HPLC assay for monitoring of TCAs in serum and plasma is characterised with external quality control data of a 3-year period. The assay is based upon single-step liquid/liquid extraction (i.e. without back extraction as employed in many other assays [11,12,16]), and the use of unmodified commercial glass vials (i.e. without silanisation of glass walls to minimise the loss of TCAs due to adsorption onto glass walls [12]). The detection limit is about 60 nM for all six compounds of interest. Despite its simplicity, the described method is demonstrated to be suitable and sufficiently reliable for diverse clinical investigations of compliance, drug overdoses, drug-induced psychoses and substance abuse. As

examples, HPLC data of specimens with suspected TCA intoxication are discussed and compared with data obtained by FPIA, a method that does not provide detailed insight into the specific TCAs present and is thus restricted to the evaluation of an overdose as recommended by the manufacturer and other laboratories [15,16].

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