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Therapeutic drug monitoring of lamotrigine using capillary electrophoresis
Evaluation of assay performance and quality assurance over a 4-year period in the routine arena

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Abstract

The performance of a capillary zone electrophoresis (CZE)-based assay for lamotrigine (LAMO) in human plasma and serum with complete internal and external quality assurance over an extended period of time is reported. The assay, originally reported by Shihabi and Oles [J. Chromatogr. B 683 (1996) 119], is based upon protein precipitation by acetonitrile and analysis of an aliquot of the acidified supernatant and was adopted in our laboratory for routine use with multi-level internal calibration on different commercial instruments. Evaluation of the calibration and control data of 103 sets of analysis and data from four years of external quality assurance based upon analysis of four-monthly sera containing LAMO and eight other anticonvulsants in sub-therapeutic, therapeutic or toxicological concentration levels revealed the robustness of the CZE-based assay and its suitability for therapeutic drug monitoring of LAMO in a routine setting. CZE data obtained in single determinations were found to compare well with the spike values and the mean of HPLC data determined in 50–70 laboratories. Furthermore, the gathered data were evaluated retrospectively using single-level internal calibration. When applied with caution, this approach was determined to produce slightly higher but otherwise equivalent drug concentrations. For the 4 years of routine operation with external quality control, the reported laboratory ranking was between 19 (out of 67 participating laboratories) and 43 (69). This is the first account of a CZE-based drug assay with complete external quality assessment.

Keywords: Quality control; Lamotrigine

1. Introduction

It is common practice that pharmacotherapy with anticonvulsants, including carbamazepine, ethosuximide, phenytoin, valproic acid, phenobarbital and lamotrigine (Lamictal, LAMO), is accompanied by therapeutic drug monitoring (TDM) [1–4]. TDM is useful for patient management and avoidance of toxicity. LAMO is one of the newer antiepileptic drugs. It is well absorbed after oral administration and about 55% is bound to serum proteins. It undergoes biotransformation by hepatic *N*-glucuronidation and elimination via the renal route. The half-life is about 25 h, is increased to an average of 60 h in presence of valproate and decreased with coadministration of enzyme inducing drugs, including carbamazepine, phenytoin and phenobarbital, to

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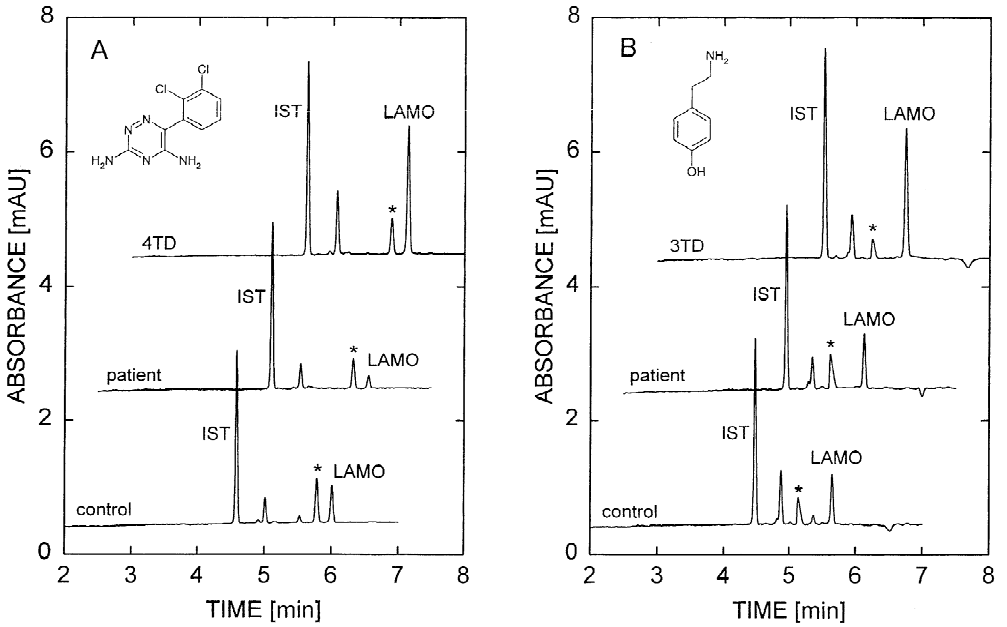

Fig. 1. Typical CZE electropherograms obtained under routine conditions using the BioFocus 3000 at a buffer pH of (A) 4.8 and (B) 4.5. Bottom graphs are data of a control plasma containing 9.75 μ*M* LAMO, center graphs are patient data (3.24 and 11.06 μ*M* LAMO, respectively) and top graphs represent external quality control data (4TD sample of 0199 and 3TD sample of 1099, respectively) with determined LAMO concentrations of 30.33 and 24.04 μ*M*, respectively. Voltage and cartridge temperature were (A) 13 kV and 20 °C, respectively, and (B) 12 kV and 25 °C, respectively. Axis scales refer to bottom electropherograms only. For the sake of presentation, center and top graphs were plotted from the previous electropherograms with *x*-axis and *y*-axis shifts of 0.5 min and 2 mAU, respectively. The chemical structures of LAMO and tyramine (IST) are given as inserts in panels (A) and (B), respectively.

an average of 15 h. Although some patients were found to tolerate LAMO concentrations >40 μ*M* without clinical toxicity, a trough level target range of about 4–16 μ*M* (1–4 μg/ml) has originally been proposed. More recently, target ranges of about 8–24 μ*M* (2–6 μg/ml) or even 12–56 μ*M* (3–14 μg/ml) are being envisaged [4–10]. LAMO is a weak base (for chemical structure refer to Fig. 1A) with a pK_a of 5.7 (value provided by its manufacturer, Wellcome Foundation, Temple Hill, Dartford, UK).

Although a radioimmunoassay [11] and an immunofluorimetric assay [12] for LAMO have been described, no convenient commercial immunoassay for LAMO has been developed and LAMO is therefore typically monitored by high-performance liquid chromatography (HPLC) [1,4–10,13–19]. Alternatively, Shihabi and co-worker reported a method based upon capillary zone electrophoresis (CZE) [10,20]. This simple assay, which is based upon protein precipitation by acetonitrile and analysis of an aliquot of the acidified supernatant using an acetate buffer at pH 4.8, has been adopted in our laboratory for routine use [21,22]. Furthermore, it has also been shown that LAMO can be analyzed together with carbamazepine, carbamazepine-10,11-epoxide and phenytoin using micellar electrokinetic capillary chromatography [23]. In this paper, the performance and long-time use of the CZE-based assay for TDM of LAMO is reported. Data and experiences gained during assay establishment on different commercial instruments and a 4-year period in the routine arena with internal and external quality control are presented and discussed. To the best of our knowledge, this is the first account of a CZE-based drug assay for which comprehensive external quality assurance is reported.

2. Materials and methods

2.1. Chemicals, quality control samples, blank matrices and preparation of calibrators and controls

All reagents were of analytical or research grade. LAMO was obtained from Wellcome Foundation (London, UK), tyramine chloride was from Fluka (Buchs, Switzerland), sodium acetate and acetic acid (100%) were from Merck (Darmstadt, Germany), and acetonitrile was from Biosolve (Valkenswaard, The Netherlands). Heathcontrol United Kingdom National External Quality Assessment Schemes (UKNEQAS) external quality control sera were purchased from Cardiff Bioanalytical Services (Cardiff, UK). UKNEQAS provides four-monthly sera referred to as 1TD, 2TD, 3TD and 4TD that contain 14 therapeutic drugs. Bovine plasma was received from the local slaughter house and was used for the preparation of calibration and control samples. Stock solutions (1 mg/ml) of LAMO and tyramine chloride [internal standard (IST)] were prepared in methanol and stored at 5 °C. The precipitation reagent was prepared by diluting 3 ml of the tyramine chloride stock solution with acetonitrile to a final volume of 100 ml. For the preparation of calibrators, the LAMO stock solution was diluted with water to reach a concentration of 50 μg/ml and 1-, 2.5-, 5-, 7.5- and 10-μl aliquots of this solution were added to 50 μl of bovine plasma producing calibrators with 3.90 μ*M* (1 μg/ml), 9.75 μ*M* (2.5 μg/ml), 19.50 μ*M* (5 μg/ml), 29.25 μ*M* (7.5 μg/ml) and 39.0 μ*M* (10 μg/ml) LAMO, respectively. Spiked bovine plasma calibrators were prepared freshly for each set of analyses. Controls were prepared batchwise. Typically, 10 ml of a control was produced via combining an appropriate amount of a diluted, independently prepared LAMO stock solution and bovine plasma and aliquots of about 150 μl were frozen in plastic vials at −20 °C. Heathcontrol UKNEQAS external quality control sera were reconstituted with water and frozen at −20 °C until analysis. All frozen samples were slowly defrosted and vortex mixed prior to analysis.

2.2. CZE instrumentation and running conditions

If not stated otherwise, CZE analyses were performed in uncoated fused-silica capillaries of 50 μm I.D.×360 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) and 34 cm (28.5 cm effective) length using the BioFocus 3000 capillary electrophoresis system (Bio-Rad Labs, Hercules, CA, USA). The capillaries were mounted in a user-assembled cartridge (Bio-Rad). Analyses were carried out with an applied voltage of 12 or 13 kV (currents: 60–70 μA), solute detection at 210 nm and hydrodynamic sample injection at 5 p.s.i.×s (1 p.s.i.= 6894.76 Pa). The temperatures of cartridge and carousel were maintained at 18–27 and 30 °C, respectively. New capillaries were conditioned with

Table 1
Conditions used for TDM of LAMO on different instruments

Instrument		Capillary					Applied power			Detection wavelength (nm)	Sample injection
Name	Manufacturer	I.D. (μm)	Total length (cm)	Effective length (cm)	Cartridge temp.[a] (°C)	Intracap. temp.[b] (°C)	Voltage (kV)	Current (μA)	Power level (W/m)		
BioFocus 3000	Bio-Rad	50	34	28.5	18–27	32–44	12 or 13	60–70	2.11–2.68	210	5 p.s.i.×s
BioFocus 2000	Bio-Rad	50	34	28.5	20	34	12	61	2.15	210	5 p.s.i.×s
P/ACE MDQ	Beckman	75[c]	37.2	27.0	25	57	12	155	5.00	210/214[d]	0.5 p.s.i., 5 s
P/ACE 5510	Beckman	75[c]	37.0	30.0	25	58	11	175	5.20	210/214[d]	0.5 p.s.i., 5 s
HP^{3D}CE	Agilent	50	48	39.8	20	54	18	65	2.44	210	50 mbar, 2 s

[a] Temperature of the circulating cooling fluid (BioFocus and P/ACE) or of the forced air (HP^{3D}CE).
[b] Intracapillary temperature estimated based upon a 6.4 and 14.0 °C temperature increase per applied 1 W/m for instruments with circulating cooling fluid and forced air temperature control, respectively [26].
[c] 50 μm I.D. capillaries could also be employed with these instruments.
[d] 210 nm with diode array detector/214 nm with selected filter of UV detector.

1 *M* NaOH (about 20 min) followed by water (about 20 min). BioFocus Integration software (version 5.0, Bio-Rad) was employed for data conversion and evaluation. The conditions employed with the BioFocus 3000 and other instruments are summarized in Table 1.

2.3. CZE assay for lamotrigine

Except for the multi-level internal calibration, LAMO was essentially determined using the CZE approach described by Shihabi and co-worker [10,20]. This assay is based upon deproteination with acetonitrile and injection of the acidified supernatant. Briefly, 50 μl of sample (control plasma, patient plasma or serum, external quality control serum) or a calibrator solution (50 μl bovine plasma fortified with LAMO as described in Section 2.1) and 100 μl of acetonitrile containing 30 μg/ml tyramine chloride were vortex mixed for about 15 s and centrifuged at 10 000 *g* for 3 min. Then, the clear supernatant was decanted and combined with 100 μl of 0.9 *M* acetic acid and an aliquot of this mixture was injected. Before each experiment the capillary was rinsed with 0.2 *M* NaOH for 1 min and running buffer for 1 min. Until 7 April, 1999, the running buffer was composed of 130 m*M* sodium acetate that was adjusted with acetic acid to pH 4.8, a pH that was suggested by Shihabi and co-worker [10,20]. Thereafter, a buffer pH of 4.5 was employed. Sets of 12–20 runs were executed and the running buffer in the inlet vial was not replenished during a set of analyses. The assay is based upon five-point internal calibration in the range of 3.9–39 μ*M* using relative peak areas (peak areas divided by detection time) for data evaluation.

2.4. HPLC assay for lamotrigine

The HPLC assay used is similar to that reported for tricyclic antidepressants [24]. Briefly, a model 510 pump (Waters, Milford, MA, USA), a model 717plus autosampler (Waters), a reversed-phase C_{18} column (Nova-Pak C_{18} 60 Å 4 μm, 150×4.6 mm, Waters) and a model UV2000 detector (Spectra-Physics, San Jose, CA, USA) were employed. The mobile phase was prepared by mixing 750 ml of 5 m*M* aqueous KH_2PO_4 buffer, 250 ml acetonitrile and 2 ml diethylamine and by adjusting the pH to 7.8 by addition of concentrated phosphoric acid. The flow-rate was 0.9 ml/min, the temperature was ambient and detection was effected at 210 or 285 nm. Methanolic standard solutions of LAMO and *N*-(1-naphthyl)ethylenediamine hydrochloride (IST) were used. Quantitation was based upon five-level internal calibration using peak areas. Calibrator (concentration range: 1.95–39.0 μ*M*) and control samples were prepared in bovine plasma. Aliquots of 0.25 ml sample (patient, calibrators and controls) were mixed with 25 μl of internal standard solution, 50 μl of 0.5 *M* NaOH and 1 ml dichloromethane containing 5% 3-methyl-2-butanol (isoamyl alcohol) for 10 min using a capped plastic tube and a shaker. After centrifugation at 9000 *g* for 3 min, the aqueous (upper) phase was discarded and the organic phase was evaporated to dryness (40 °C under air) and reconstituted in 200 μl methanol. For analysis, aliquots of 30 μl were injected. Extraction recovery for LAMO and *N*-(1-naphthyl)ethylenediamine (at 5 μg/ml each) were determined to be 87 and 86%, respectively ($n=2$). Calibration graphs for LAMO were linear ($r>0.999$; $F>1000$ ($P<0.0001$)) and characterized with very small *y*-intercepts. The detection limit for LAMO was determined to be 0.4 μ*M*. LAMO and IST eluted after about 4.7 and 9.7 min, respectively (data not shown). Because of insufficient long-term stability of *N*-(1-naphthyl)-ethylenediamine in solution, it was later substituted by 4′-bromoacetanilide.

2.5. Statistical and graphical data analysis

Comparative drug levels were analyzed by linear regression analysis and by bias analysis defined as the mean and standard deviation of the differences of each data pair [25]. Comparative sets of data were statistically compared using the Mann–Whitney rank sum test and each set of data was subjected to the normality test. Comparison of three groups of data was performed with the Kruskal–Wallis one-way analysis of variance on ranks test. Statistical evaluations were performed with SigmaStat for Windows version 1.0 (Jandel, Corte Madera, CA, USA) and graphical presentations were made with SigmaPlot version 2.01 (Jandel).

3. Results and discussion

3.1. Method selection and general comments about the CZE assay

In most laboratories, LAMO plasma and serum levels are being determined by HPLC [1,4–10,13–19]. Thus, when asked to provide a TDM service for LAMO, our laboratory evaluated an HPLC method (Section 2.4) and a CZE assay based upon the work of Shihabi and co-worker [10,20]. The two methods were found to provide comparable drug levels and comparable assay performance in terms of imprecision. For a set of 24 external quality control sera, linear regression analysis revealed a linear correlation ($r=0.976$). The two methods, however, were noted to differ in sample treatment (drug extraction versus protein precipitation) and therefore lab technician time, the requirement of large amounts (HPLC) versus small amounts (CZE) of organic solvents and sample throughput (duration of an analytical run including reconditioning of the column of 13 vs. 9 min for HPLC and CZE, respectively). Thus, based upon a careful evaluation, it was decided to introduce the CZE assay into the routine arena in November 1997 and we are now in the position to report a complete assay performance and quality assurance from a 4-year time period.

In the CZE assay, LAMO is determined after protein precipitation with acetonitrile and analysis of an aliquot of the acidified supernatant using a 130 m*M* acetate buffer. Based upon the acetonitrile content of the injected sample, analytes are being stacked, this providing the required sensitivity for this drug despite its 3-fold initial dilution with the precipitation agent [10]. Typical electropherograms obtained with bovine plasma-based controls, patient samples and external quality control sera are presented in Figs. 1 and 2. Employing a pH 4.8 buffer, as was originally proposed by Shihabi and Oles [10,20], provided data with an endogenous compound migrating shortly ahead of LAMO (peak marked with an asterisk in Fig. 1A). Based upon the absorbance spectra gathered (data not shown), all three samples appear to comprise the same peak. Furthermore, it can reasonably be assumed that this

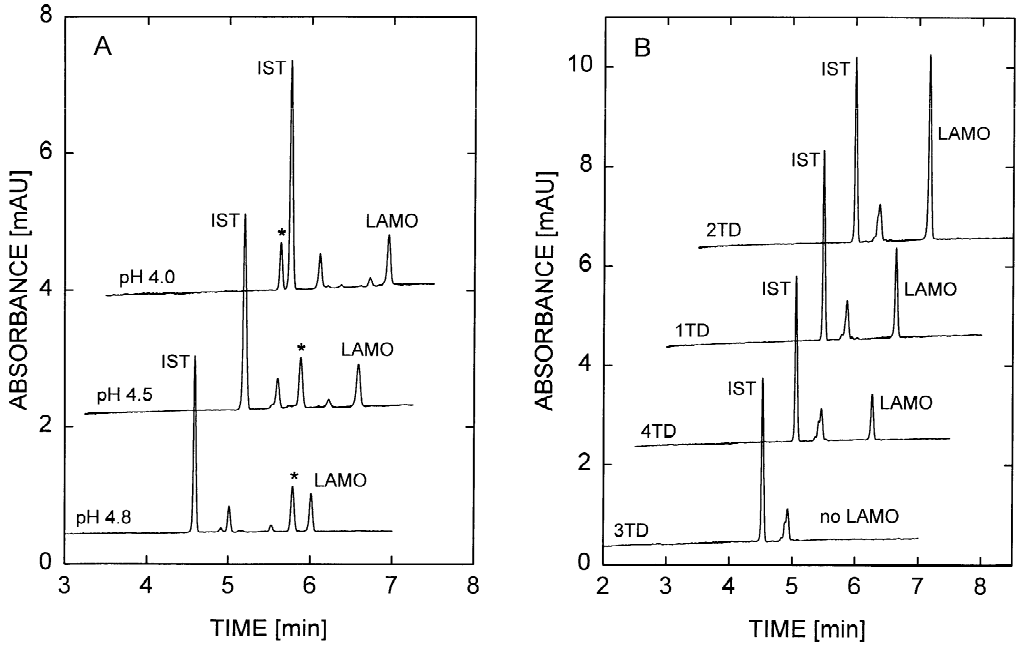

Fig. 2. CZE electropherograms of (A) a control plasma containing 9.75 μ*M* LAMO analyzed in buffers with different pH values (voltage/temperature: 13 kV/20 °C, 12 kV/18 °C and 12 kV/18 °C, from bottom to top, respectively) and (B) the four external quality control sera of 0899 analyzed at the same conditions as for Fig. 1B.

is the same peak as that observed shortly behind LAMO in the electropherograms of Shihabi and Oles [10,20]. Although this peak was found to be nicely separated from LAMO, its presence was irritating. As detection times and buffer conditions were somewhat unstable (see below), unambiguous interpretation of electropherograms containing small (<10 μM) amounts of LAMO (example: patient electropherogram in Fig. 1A) was not straightforward. Changes of buffer pH were found to provide large shifts of the peak marked with the asterisk (Fig. 2A). In order to avoid an interference with tyramine (IST) and LAMO, the buffer pH was changed to 4.5 effective 7 April 1999. Typical electropherograms obtained with a buffer pH of 4.5 are depicted in Fig. 1B. Furthermore, in certain sets of electropherograms, several of the endogenous compounds were found to comigrate (Fig. 2B). Slight differences in pH stemming from buffer preparation (calibration of pH meter) and from a possible carry over of NaOH from the reconditioning step between runs are assumed to be responsible for the pattern changes. The latter effect is illustrated with the calibration data presented in Fig. 3, data that were registered in the order of decreasing LAMO content (from top to bottom electropherogram). The endogenous compound marked with an asterisk is shown to shift towards the LAMO peak, an indication that the pH is slightly increasing from run to run. After a few runs, the pattern became stable (Fig. 1B). This behavior which is presumably associated with the electrode being in too close proximity of the capillary was observed occasionally only and had no effect on the detection times of the solutes of interest. For the entire set of data (total of 15 electropherograms), relative standard deviation (RSD) values of the detection times of LAMO, IST and LAMO/IST ratio were determined to be 0.91, 0.63 and 0.32%, respectively. Furthermore, at the beginning of the registration of a new set of data, markedly increasing detection times were often observed for the first three runs, a phenomenon that was previously also mentioned by Shihabi [20] and was found to be independent of the buffer pH. Thus, each set was typically commenced by triplicate analysis of the 39 μM calibrator.

During the assay evaluation in 1997 and the 4 years in the routine laboratory, most runs were

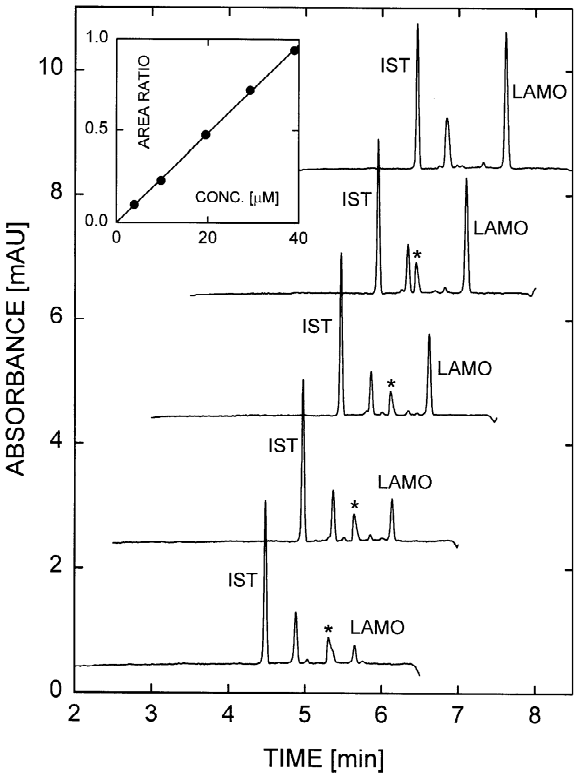

Fig. 3. CZE electropherograms of the five calibrators obtained on the BioFocus 3000 using a buffer pH of 4.5. The data are from the same set as those of Fig. 1B. For the sake of presentation, electropherograms from bottom (lowest calibrator) to top (highest calibrator) were plotted with x-axis and y-axis shifts of 0.5 min and 2 mAU, respectively. The insert depicts the calibration graph characterized by $y=0.02422x+0.0013$ and $r=0.9995$.

performed on the Bio-Focus 3000 (for exceptions see below) using a total of six capillaries. As the room temperature in the laboratory was often 25 °C or higher and the Bio-Focus 3000 had difficulties in maintaining the capillary temperature of 20 °C, the cartridge temperature was increased to 25 °C (between 2 August 1999 and 5 September 2000) and finally to 27 °C. Furthermore, although a voltage of 12 kV was typically applied, selected sets of data were collected with 13 kV. These changes, the adjustment of buffer pH discussed above and small differences in capillary conditioning between sets of data had an impact on detection times. Detection times for LAMO and IST were found to range from 4.5–6.9 and 3.5–5.3 min, respectively. Ratios of detection times (LAMO/IST), however, varied be-

tween 1.21 and 1.34 only. Evaluation of the control data of 1998, 1999 and 2000 ($n=71$) revealed RSD values of 9.15, 8.45 and 2.07% for the detection time of LAMO, the detection time of the IST and the detection time ratio, respectively.

During the course of our work, the LAMO CZE assay was adapted to different CZE instruments and the conditions employed are summarized in Table 1. All experiments were performed at field strengths between 320 and 380 V/cm, estimated intracapillary temperatures of 32–58 °C and 6.0–7.0 min electropherogram registration time intervals. A total of 103 sets of data were generated (Table 2) with 84 sets being performed on the BioFocus 3000, three on the BioFocus 2000 (in 1998), 13 on the P/ACE MDQ (two in 1999 and 11 in 2001) and three on the $HP^{3D}CE$ (in 1998). The entire experimental work was done by two lab technicians.

3.2. Multi-level calibration and internal quality control

Table 2 summarizes the analytical characteristics of the five-level internal calibration. All calibration graphs were found to be linear ($r>0.9902$) with F values >100 ($P\leq 0.0097$). For the assay development in 1997 and the entire 4-year period during which the CZE assay was employed in the routine arena, mean r values were determined to be ≥ 0.9980 and F values were typically >1000 ($P<0.0001$). Furthermore, the y-intercepts were observed to be significantly smaller than the ratio produced by the smallest calibrator and were negligible in most cases. The detection limit was determined to be 2 μM (BioFocus 3000), the limit for quantitation was taken as 3 μM and patient samples producing a result between 2 and 4 μM were reported as <4 μM. Throughout this work, samples were analyzed only once. Analysis of 102 patient samples revealed LAMO values between 0 and 130 μM (mean: 18.6 μM; median: 10.2 μM). Seventy-eight results were within the quantification range of 3–40 μM (mean: 12.40 μM; median: 9.94 μM), 15 samples had values between 41.2 and 130 μM (mean: 61.6 μM, median: 61.3 μM) and nine patient sera were found to have a LAMO concentration <3 μM.

Typical assay imprecision data are summarized in Table 3. For a LAMO concentration of 9.75 μM, intra-day and inter-day reproducibilities are shown to be about 1.5 and 5.3%, respectively. Internal quality control could be assessed over a 4-year time period. For the first 3 years during which a total of 71 sets of data were analyzed, a sample containing 9.75 μM LAMO from three different batches (batch changes: 18 September 1998 or set 17 of Fig. 4A and 11 October 1999 or set 44 in Fig. 4A) was used as control sample and was analyzed following the five calibrators. The changes of the batches are not obvious from the data presented in Fig. 4A. All controls were essentially found to provide LAMO concentrations within the target range defined as drug level $\pm 10\%$ (Table 3, Fig. 4A). A total of eight values were noted to be out of that range but well within $\pm 20\%$ (Table 3). RSD values calculated for

Table 2
Statistical evaluation of calibration data[a]

	n[b]	Slope		y-Intercept		r			F value	
		Mean $(\mu M)^{-1}$	RSD (%)	Mean	RSD (%)	Range	Mean	RSD (%)	F	P
Interday reproducibility[c]	5	0.0207	7.91	0.016	554.4	0.9949–0.9999	0.9987	0.22	≥1192	<0.0001
Interday reproducibility[d]	15	0.0205	7.90	0.022	656.7	0.9913–1.0000	0.9980	0.26	≥337	≤0.0004
Routine year 1998	29	0.0197	11.47	0.012	176.4	0.9903–1.0000	0.9987	0.20	≥102	≤0.0097
Routine year 1999	18	0.0223	10.85	0.010	115.4	0.9969–0.9999	0.9990	0.09	≥318	≤0.0031
Routine year 2000	24	0.0221	8.91	0.023	157.8	0.9913–1.0000	0.9986	0.18	≥170	≤0.0010
Routine year 2001	17	0.0212	7.70	−0.003	834.9	0.9959–1.0000	0.9989	0.12	≥360	≤0.0003

[a] The concentration values and peak area ratios were taken as x-axis and y-axis, respectively.
[b] Number of repeats or number of data sets.
[c] Data obtained during development of the assay.
[d] Data of 1997.

Table 3
Imprecision data and statistical evaluation of internal quality control data

	n[a]	Drug level (μM)	Mean (μM)	RSD (%)	Number of values outside target range[b]
Intraday reproducibility	5	9.75	9.56	1.54	0
Interday reproducibility	5	9.75	9.28	5.27	1
Interday reproducibility[c]	15	9.75	9.15	6.37	5
Routine year 1998	29	9.75	9.76	6.88	4
Routine year 1999	18	9.75	9.75	5.15	0
Routine year 2000	24	9.75	10.07	5.74	4
Routine year 2001	14[d]	8.00	7.59	8.41	4
Routine year 2001	14[d]	18.00	18.02	6.90	2
Routine year 2001	14[d]	38.00	42.23	9.63	4 (1)
Single-level calibration 1998	29	9.75	10.15	7.20	8 (1)
Single-level calibration 1999	18	9.75	10.14	7.27	6
Single-level calibration 2000	24	9.75	10.72	8.40	10 (2)

[a] Number of repeats or number of data sets.
[b] Target range was taken as ±10% of drug level. Numbers in brackets indicate number of values outside±20%.
[c] Data of 1997.
[d] Three sets were undertaken with single-level control (9.75 μM).

each year were found to be ≤7% (Table 3). For the 3-year period, mean, SD and RSD were calculated to be 9.86 μM, 0.611 μM and 6.20%, respectively (Fig. 4A). Multi-level internal quality control was employed during the fourth routine year for which similar data were obtained. All these data compare favorably with the specifications of drug assays based on immunoassays, HPLC or GC.

3.3. Quality assessment with external samples and laboratory ranking

The purchased quality control samples referred to as UKNEQAS therapeutic drugs mixture are prepared from human serum containing 14 analytes, namely nine antiepileptics (carbamazepine, carbamazepine-10,11-epoxide, ethosuximide, LAMO, phenytoin, primidone, valproic acid, clonazepam and phenobarbital), theophylline, caffeine, gentamicin, digoxin and lithium. These analytes are present in sub-therapeutic, therapeutic and toxic concentration levels (Table 4). With the CZE assay for LAMO, very simple electropherograms are obtained (Figs. 1 and 2). For the presented examples, CZE with acetonitrile deproteination is demonstrated to provide results that are within the ±10% range of the spike value and that are close to the consensus mean of drug concentrations that were mainly determined by HPLC (Table 4). The other antiepileptics, theophylline, caffeine and gentamicin appear not to interfere with LAMO under the chosen experimental conditions. This was particularly noted for all sera containing no LAMO (for an example refer to the bottom graph of Fig. 2B).

A total of 288 quality control samples purchased between 1996 and 2001 were analyzed. LAMO in the sera of 1996 and most of those of 1997 was determined retrospectively in batches of about 20 samples. Commencing in November 1997, the four-monthly samples were analyzed once a month together with patient sera and controls and the data were reported to the Heathcontrol external quality assessment scheme in Cardiff (UK). Except for two outliers with increased concentration (>40 μM), LAMO concentrations were found to be within (n= 248) or below (n=38) the range of 3–40 μM in which quantitative data were generated. Drug levels of the 248 sera obtained by CZE were compared to the reported data graphically (Fig. 5) and statistically (Table 5). The CZE data included in this evaluation are not in complete agreement with those reported to the scheme. Results which were wrongly tran-

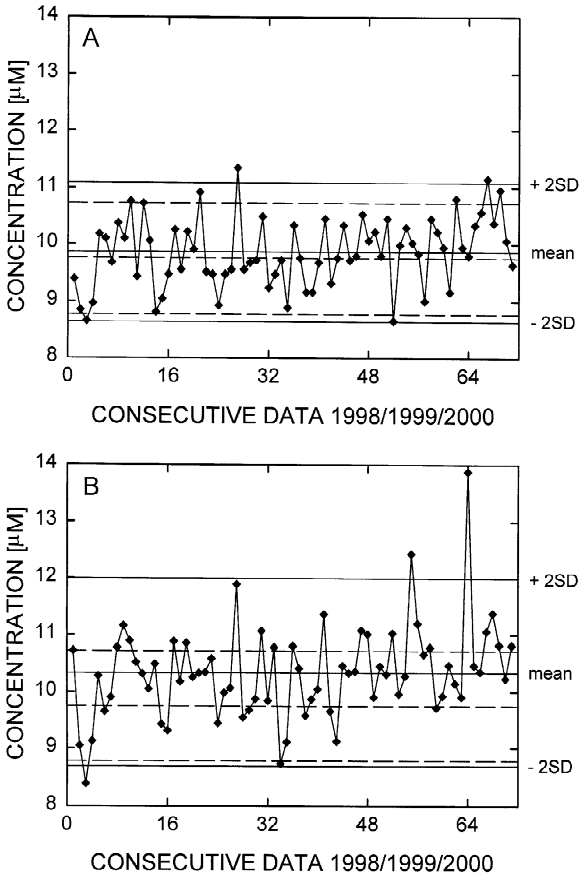

Fig. 4. Internal quality control data over a 3-year period during which a total of 71 sets of samples were analyzed with data evaluation based upon (A) five-level internal calibration and (B) single-level internal calibration. The solid lines represent mean and mean±2 SD of the experimental data, whereas the broken lines represent the target drug concentration (9.75 μ*M*, center line) and target drug level ±10% (upper and lower lines, respectively).

scripted, miscommunicated or wrongly evaluated (see below) were corrected prior to data evaluation. The CZE data were compared with the reported spike values and with the mean of the data obtained in other laboratories (denoted as consensus mean, mean of data that were mainly monitored by HPLC). For all sets of data, mean and median values were found to be different and normality tests failed ($P<0.0001$). Statistical analysis using the Mann–Whitney rank sum test revealed the absence of a statistically significant difference between each pair of input groups ($P>0.27$). The same was found to be true with the Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks test for the comparison of each set of three input groups ($P>0.36$). Furthermore, linear regression analysis of comparative data pairs revealed linear relationships ($r\geq0.986$) with relatively small *y*-intercepts and slopes that were reasonably close to unity (Table 5). Graphs comparing the CZE data of the 248 samples with the corresponding consensus mean values and spike values are presented in Figs. 5A and 5B, respectively. Graphs with single annual data sets were found to be very similar and are thus not depicted. Furthermore, the bias expressed by the mean of the differences of the data pairs was found to be small for all cases, this particularly in relation to the concentration range monitored (Table 5). It was interesting to find that the bias between CZE and spike data was always negative, indicating that the CZE data were on average slightly smaller. With the exception for the data of 1997, positive bias values were noted for the comparison of CZE and consensus mean data (Table 5). Furthermore, plotting the difference against the mean of corresponding data pairs provided deeper insight into the equality of two sets of data. A graph for the CZE and consensus mean data is presented in Fig. 6A. Although the mean of the differences was found to be small (0.343 μ*M*, Table 5), one could reach the conclusion that there is better agreement for drug levels < about 20 μ*M*. That this is not the case is shown with the graph depicted in Fig. 6B in which the relative differences (in %) were plotted against the mean of the same 248 data pairs.

The data of 38 sera could not be included in this evaluation as they contained LAMO concentrations <3 μ*M*. Ten of those samples had no LAMO at all. CZE was found to correctly identify these samples (example: Fig. 2B). As the LAMO concentrations of the two outliers (one from 1996 and one from 2000) were above the calibration range (45.5 and 49.1 μ*M*; 48.0 and 43.8%, respectively, higher than the corresponding consensus mean values), the origin of the increased concentration was not further investigated.

For all the participants of the Heathcontrol external quality control scheme, the performance of the laboratory is assessed monthly. Results obtained include a frequency diagram for each sample (Fig. 7A), a line graph which presents a linear regression

Table 4
Drug levels (in μ*M*) of external quality control sera whose electropherograms are presented in Figs. 1 and 2

Drug	Type of value[a]	4TD0199 (Fig. 1A)	3TD1099 (Fig. 1B)	1TD0899 (Fig. 2B)	2TD0899 (Fig. 2B)	3TD0899 (Fig. 2B)	4TD0899 (Fig. 2B)
LAMO	CZE	30.33	24.04	18.32	39.06	0.00	10.32
LAMO	Consensus mean	31.03	22.77	17.43	36.87	0.57	10.10
LAMO	Spike	33.30	24.73	18.91	39.75	0.00	11.12
Phenytoin	Spike	24.8	51.7	71.4	138.5	20.1	47.5
Phenobarbital	Spike	137.5	231.7	20.5	180.6	90.0	220.2
Carbamazepine	Spike	5.0	30.0	42.5	60.3	0.00	25.5
Carbamazepine-10,11-epoxide	Spike	6.5	3.1	8.9	12.9	0.00	6.0
Valproic acid	Spike	99.5	1301.2	561.1	101.9	1208.8	443.2
Primidone	Spike	7.4	44.7	27.4	0.00	79.3	44.7
Clonazepam	Spike	0.151	0.000	0.084	0.199	0.321	0.102
Ethosuximide	Spike	638.7	419.1	359.6	797.9	0.00	630.3
Theophylline	Spike	98.2	139.6	58.1	105.0	64.5	149.2
Caffeine	Spike	4.9	10.0	53.6	39.5	5.0	99.4
Gentamicin	Spike	3.9	24.0	5.0	1.6	14.1	29.9

[a] CZE refers to the drug level determined by CZE, consensus mean represents the mean of data reported by 50–56 laboratories (independent of analytical method used) and spike is the reported level of serum fortification.

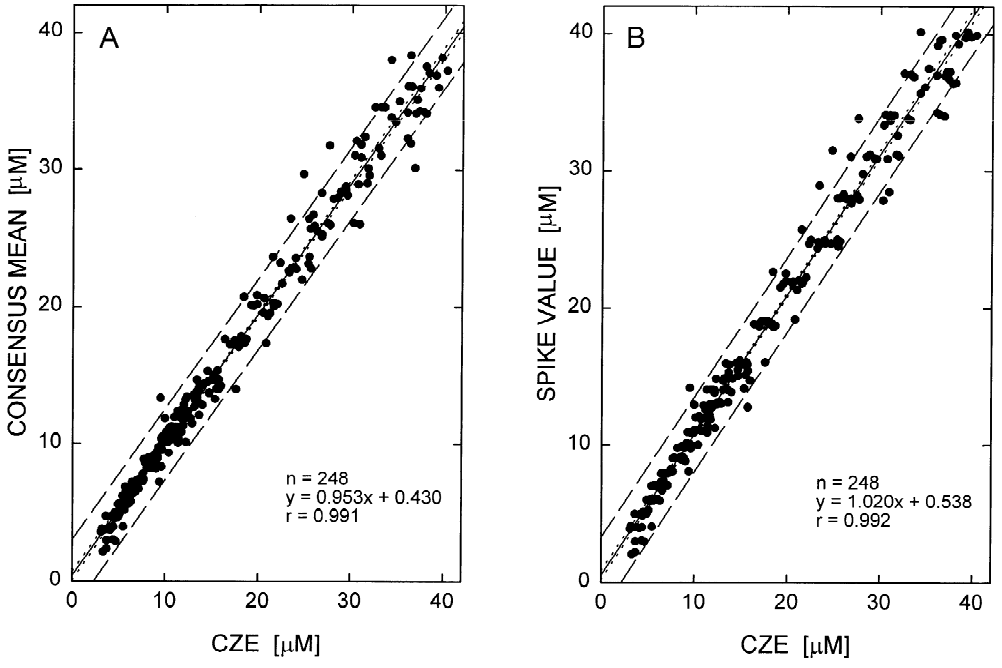

Fig. 5. Comparative LAMO drug levels for 248 quality control sera with (A) CZE versus reported consensus mean data and (B) CZE versus reported spike data. The solid lines represent correlation graphs determined by linear regression analysis, the broken lines describe the 95% prediction interval around the regression line and the dotted lines are the 95% confidence interval for the regression line (for statistical data see Table 5).

Table 5
Laboratory ranking, statistical, linear regression analysis and bias analysis data of comparative LAMO drug levels in external quality control samples

Year	Ranking/number labs[a]	n[b]	CZE data		Reported data of quality assurance scheme or CZE_{ML} data[c]			Linear regression analysis data[d]			Bias analysis data[e]	
			Mean (μ*M*)	Median (μ*M*)		Mean (μ*M*)	Median (μ*M*)	Slope	*y*-Intercept (μ*M*)	*r*	Mean (μ*M*)	SD (μ*M*)
1996	–	44	15.3	11.9	Spike value	15.9	12.7	1.064	−0.342	0.996	−0.637	1.18
					Consensus mean	14.8	11.3	0.984	−0.223	0.996	0.471	0.88
1997	–	41	15.9	12.2	Spike value	17.6	14.0	1.110	−0.040	0.986	−1.716	2.15
					Consensus mean	16.6	13.3	1.050	−0.140	0.989	−0.681	1.61
1998	24/56	41	17.1	13.6	Spike value	17.7	13.9	0.993	0.667	0.996	−0.551	1.02
					Consensus mean	16.4	12.8	0.926	0.555	0.995	0.720	1.33
1999	19/67	41	16.6	13.1	Spike value	17.7	14.7	1.017	0.847	0.995	−1.130	1.06
					Consensus mean	16.3	13.2	0.952	0.552	0.996	0.244	1.00
2000	43/69	41	16.3	12.4	Spike value	16.7	13.1	0.981	0.755	0.995	−0.441	1.14
					Consensus mean	15.8	11.9	0.900	0.604	0.994	1.030	1.50
2001	35/72	40	17.1	13.7	Spike value	18.1	14.9	0.987	1.25	0.993	−1.016	1.22
					Consensus mean	16.8	13.7	0.922	1.070	0.993	0.266	1.42
1996–2001	–	248	16.4	12.8	Spike value	17.3	14.0	1.020	0.538	0.992	−0.911	1.40
					Consensus mean	16.0	12.8	0.953	0.430	0.991	0.343	1.40

[a] Monthly reporting was commenced 11/97 and ranking is based upon the 12-month summary reports provided in January of each following year.
[b] Number of samples whose CZE data were within 3–40 μ*M*.
[c] The consensus mean represents the mean of data reported by 50–70 laboratories (independent of analytical method used).
[d] Our data and reported data were taken as *x*-axis and *y*-axis, respectively.
[e] To analyze the bias, the mean and SD of the difference of each data pair (CZE value—reported value) was calculated.

of the data for the current month which attempts to identify systematic errors [27] (Fig. 7B) and a 12-month bias index score (BIS) summary (Fig. 7C). Data >3 SD from the consensus mean are considered outliers and are excluded in the evaluation, data outside the quantitation range of the assay are listed as out of range, no data or not detectable. The BIS is the difference of the laboratory measurement from the consensus mean scaled in terms of a chosen coefficient of variation×100 [28]. For the 1999 time period, one serum did not contain any LAMO, three sera had LAMO concentrations <3 μ*M* and could thus not be measured with our CZE assay, and one-monthly set of data was reported in the wrong concentration units (μg/ml instead of μ*M*). For the latter mistake, three values were listed as out of range and one as rejected. Thus, for 1999, The BIS summary includes a total of 42 results (Fig. 7C). Furthermore, a 12-month summary report with a ranking score is provided twice a year. Panel D of Fig. 7 depicts the graph for 1999, a time period during which our laboratory ranked 19th out of 67 laboratories participating in the external quality control scheme for LAMO. The scores for all 4 years are listed in Table 5.

In conclusion, all these data illustrate the excellent performance of the CZE assay for LAMO. The data obtained from the monthly external quality control compare favorably with those generated by HPLC in other laboratories (Table 5). It is hoped that the CZE assay with external quality assessment will be adopted by other laboratories such that this analytical system can further be evaluated with data from different laboratories as has been done for other drugs using immunoassays, HPLC or GC [29–31].

3.4. Data evaluation based upon single-level internal calibration

Although not common in clinical settings in Europe and thus not applied in our laboratory, Shihabi and co-worker suggested single-level calibration with the 39.0 μ*M* calibrator and an independent control [10,20]. In a retrospective action,

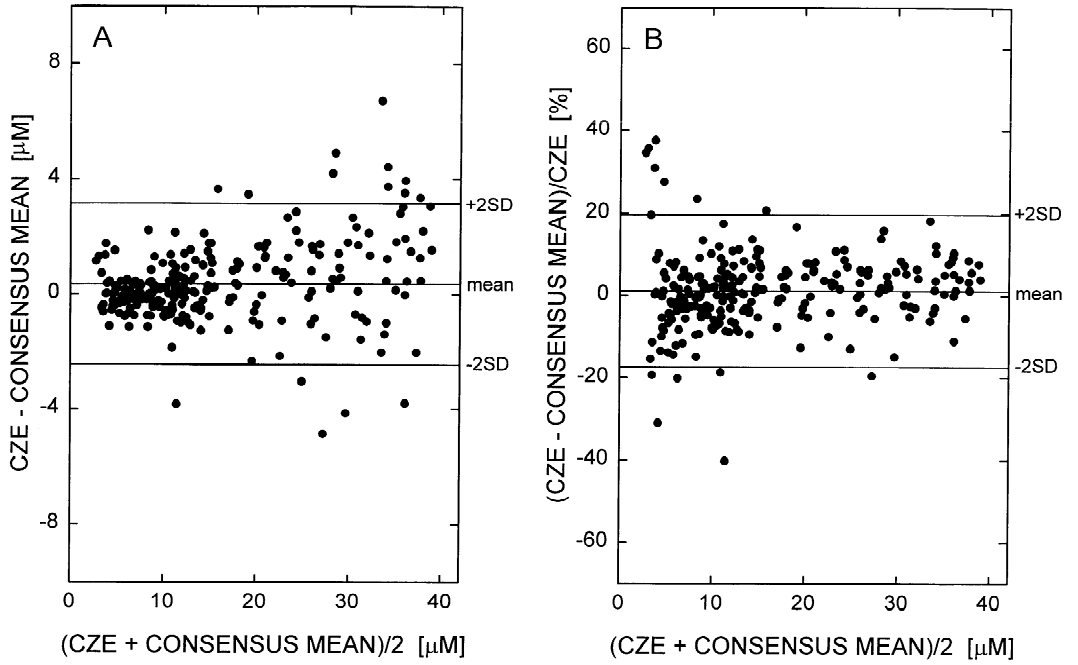

Fig. 6. Bias analysis data of 248 quality control sera with (A) difference versus mean of each CZE and consensus mean data pair (for statistical data see Table 5) and (B) relative difference versus mean of each CZE and consensus mean data pair. The solid lines represent mean and mean±2 SD of the data.

the routine data of 1998–2000 with its 71 sets of analyses were reevaluated employing the highest calibrator (39 μ*M* LAMO) for calibration and the 9.75 μ*M* control. For the 3 years and the whole set of 71 values, RSD values for the peak area ratio of the calibrator were calculated to be 12.58, 10.94, 7.30 and 12.08%, respectively. Similar area ratio data were obtained for the control whose calculated mean concentration and RSD values are presented in Table 3. The mean and RSD of the 71 control values were calculated to be 10.33 μ*M* and 8.02%, respectively. Compared to multi-level calibration, mean and RSD values were found to be higher. It is thus not surprising that more values were found to be above the anticipated target range. With the exception of the very high values (sets 55 and 64), the overall distribution (Fig. 3B) was found to be similar to that obtained with multi-level calibration (Fig. 3A). The RSD for the 69 values was calculated to be 6.55%, a value that compares well to the 6.20% obtained for the case with multilevel calibration. The mean of the 69 control values, however, remains slightly higher (10.26 vs. 9.86 μ*M*). The two very high values stemmed from sets for which multi-level calibration graphs had relatively large *y*-intercepts (0.0865 and 0.1012, respectively). Thus, these data suggest that single-level calibration is less robust than multi-level calibration as discussed above.

Drug levels of the 123 quality control sera obtained by CZE with single-level calibration (CZE_{SL}) were compared to the reported data and CZE data determined by multi-level calibration (CZE_{ML}) (Table 6, Fig. 8). For all sets of data, mean and median values were found to be different and normality tests failed ($P<0.0001$). Statistical analysis revealed the absence of a statistically significant difference between each pair of input groups and each set of four input groups. Furthermore, linear regression analysis of comparative data pairs revealed linear relationships ($r\geq0.991$) with relatively small *y*-intercepts and slopes that were reasonably close to unity (Table 6). Graphs comparing the CZE_{SL} data of the 123 samples with the corresponding consensus mean values and CZE_{ML} data are presented in Figs. 8A and 8B, respectively. Graphs with single annual data sets were found to be

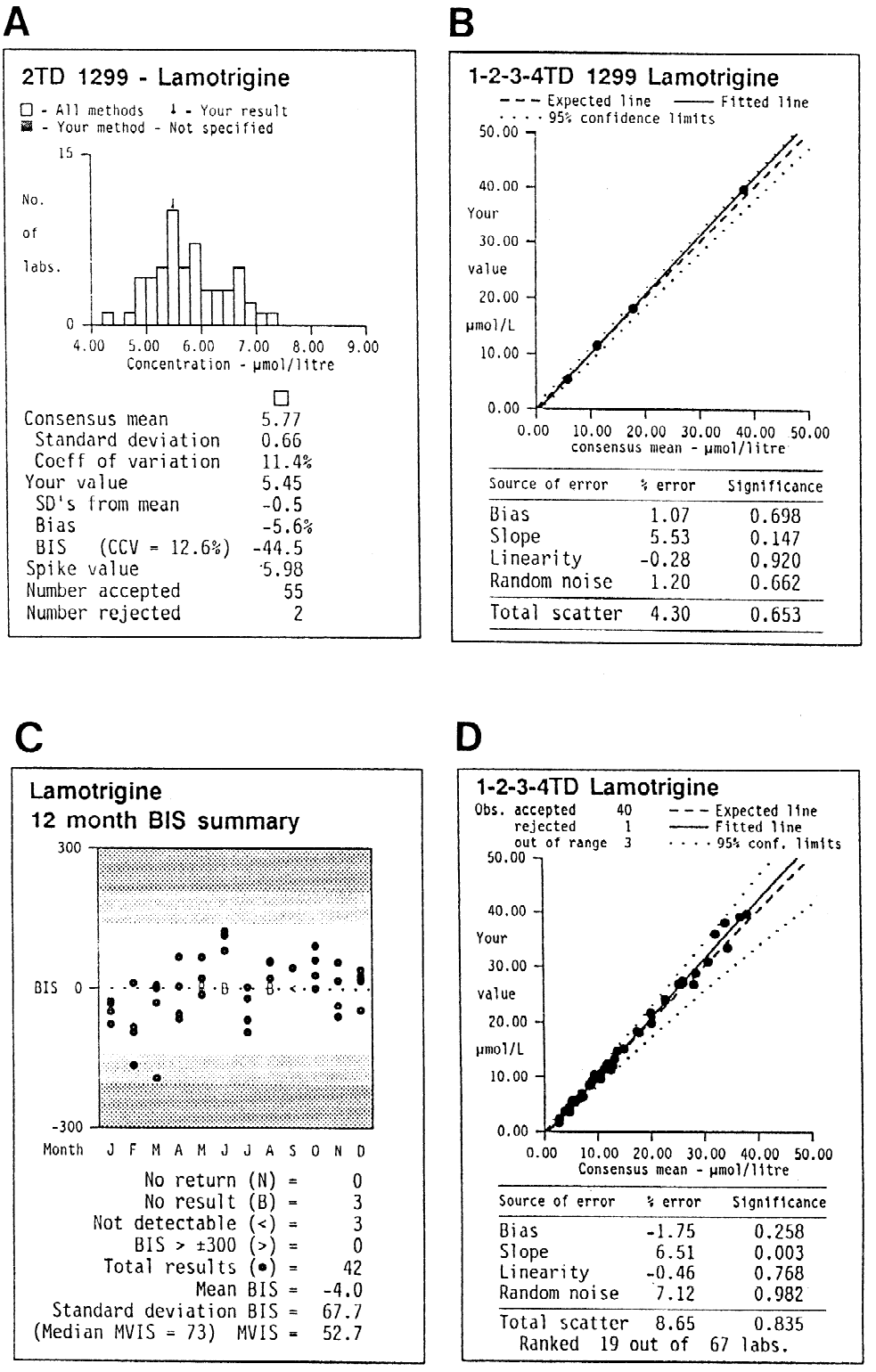

Fig. 7. Typical examples of data reports from the Heathcontrol external quality control scheme representing (A) the frequency diagram for the second sample of December 1999, (B) the line graph which presents a linear regression of the four data of December 1999, (C) the 1999 BIS summary and (D) the 1999 performance summary with the laboratory ranking.

Table 6
Statistical, linear regression analysis and bias analysis data of LAMO drug levels determined by single-level calibration[a]

Year	*n*	CZE_{SL} data		Reported data of quality assurance scheme or CZE_{ML} data[b]			Linear regression analysis data[c]			Bias analysis data[d]	
		Mean (μ*M*)	Median (μ*M*)		Mean (μ*M*)	Median (μ*M*)	Slope	*y*-Intercept (μ*M*)	*r*	Mean (μ*M*)	SD (μ*M*)
1998	41	17.5	14.0	Spike value	17.7	13.9	0.992	0.344	0.993	−0.212	1.305
				Consensus mean	16.4	12.8	0.924	0.265	0.991	1.059	1.586
				CZE_{ML}	17.1	13.6	1.000	−0.353	0.999	0.331	0.541
1999	41	16.8	13.3	Spike value	17.7	14.7	1.020	0.533	0.993	−0.851	1.266
				Consensus mean	16.3	13.2	0.954	0.249	0.995	0.522	1.141
				CZE_{ML}	16.6	13.1	1.000	−0.326	0.999	0.279	0.492
2000	41	16.9	13.3	Spike value	16.7	13.1	0.985	0.082	0.992	0.173	1.394
				Consensus mean	15.8	11.9	0.904	−0.013	0.991	1.644	1.643
				CZE_{ML}	16.3	12.4	1.010	−0.708	0.998	0.173	0.626
1998–2000	123	17.1	13.4	Spike value	17.4	14.0	0.998	0.332	0.992	−0.297	1.379
				Consensus mean	16.0	12.7	0.927	0.178	0.991	1.075	1.532
				CZE_{ML}	16.7	13.1	1.000	−0.464	0.999	0.411	0.570
1998–2000[e]	123	16.7	13.1	Consensus mean	16.0	12.7	0.925	0.579	0.994	0.664	1.320

[a] Retrospective data evaluation of the 1998–2000 samples of Table 5.
[b] The consensus mean represents the mean of data reported by 50–70 laboratories (independent of analytical method used).
[c] CZE_{SL} data were taken as *x*-axis.
[d] To analyze the bias, the mean and SD of the difference of each data pair (CZE_{SL} value—reported or CZE_{ML} value) was calculated.
[e] CZE_{ML} instead of CZE_{SL} data.

very similar and are thus not depicted. Furthermore, the bias expressed by the mean of the differences of the data pairs was found to be small for all cases (Table 6) and plotting the difference against the mean of corresponding data pairs provided deeper insight into the equality of two sets of data. Graphs for the consensus mean and CZE_{ML} data are presented in Fig. 8C,D, respectively. These data reveal that CZE_{SL} data are slightly higher and that there is an excellent agreement between the CZE_{SL} and CZE_{ML} data. Based on the control values (Fig. 3B), the latter aspect could be regarded as a surprise. However, no external quality control sera were analyzed on the 2 days with the high *y*-intercepts (see above). Thus, these data suggest that single-level calibration can indeed be used when applied with caution.

4. Conclusions

The performance of the CZE-based assay with multi-level internal calibration was assessed with calibration and control data of 103 sets of analysis performed by two technicians within about 4.5 years and complete external quality assurance over a 4-year period. The assay is shown to be simple, inexpensive, robust and executable on different commercial instruments. CZE data of external quality control sera were found to compare well with the spike values and the consensus mean of drug levels generated by HPLC in 50–70 other laboratories. Furthermore, a retrospective evaluation with single-level calibration (as originally suggested by Shihabi and co-worker [10,20]) is demonstrated to provide slightly higher but otherwise identical LAMO concentrations. For cases in which the multi-level calibration graphs had a significant *y*-intercept, however, single-level calibration data were found to strongly deviate. Thus, single-level calibration should be used with caution. To our knowledge, this is the first paper reporting CZE-based TDM data from the routine arena with complete internal quality control and external quality assurance via analysis of four monthly external quality control samples whose data were directly reported to the quality control scheme. For the 4 years under routine operation, the laboratory ranking was 24 (out of a total of 56 particip-

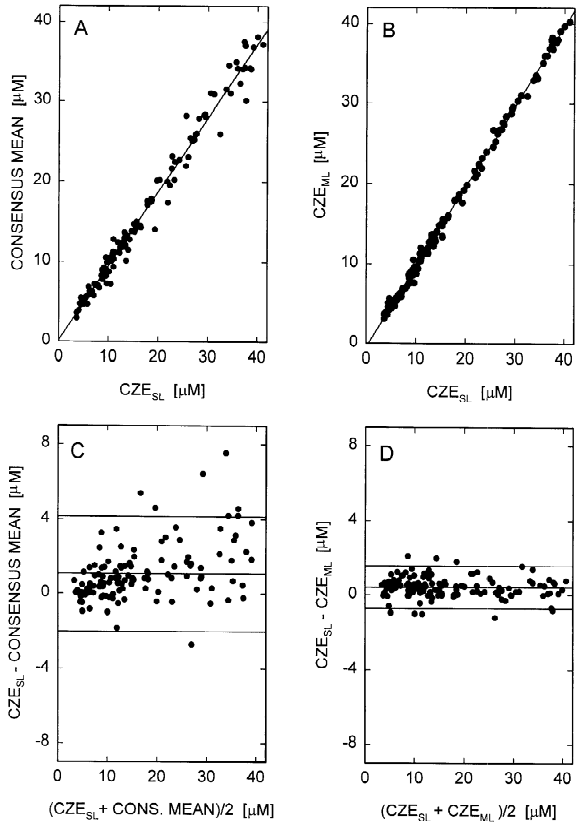

Fig. 8. Comparison of LAMO drug levels of 123 external quality control sera determined by CZE_{SL} with (A,C) consensus mean data and (B,D) concentrations monitored with CZE_{ML}. The straight lines in panels (A) and (B) are linear regression lines and those in panels (C) and (D) represent the mean (center line) and mean±2 SD of the differences of the data pairs. For values and coefficients refer to Table 6.

ants), 19 (67), 43 (69) and 35 (72) with an average ranking of 45.6%. The presented data further broadens the confidence in CZE technology for the TDM laboratory.

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