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Liquid chromatography and capillary electrophoresis analysis of polyanionic quinobene*

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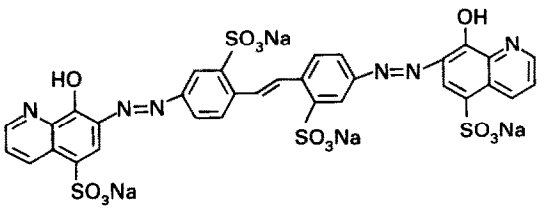
Abstract: Quinobene is the tetrasodium salt of an organic tetrasulphonic acid. Its unusual solubility characteristics makes the development of LC analysis difficult. However, a specific, precise and accurate LC assay was eventually achieved for quinobene. The assay required gradient elution and was not efficient for quinobene with respect to plate number. As an alternative, a capillary electrophoresis (CE) assay was also developed for quinobene. The CE assay was comparable to the LC assay in precision and accuracy. It was unaffected by the unusual solubility characteristics of quinobene and was more specific, efficient and rugged than the LC assay.

Keywords: *Quinobene; liquid chromatography; capillary electrophoresis analysis.*

Introduction

Quinobene, the tetrasodium salt of 4,4′-bis(8-hydroxy-5-sulpho-7-quinolineazo)-stilbene-2,2′-disulphonic acid (Fig. 1), is prepared from 4,4′-diaminostilbene-2,2′-disulphonic acid and 8-hydroxyquinoline-5-sulphonic acid by a diazotization process accompanied by a coupling reaction [1, 2]. It belongs to the group of sulphonated dyes that inhibit HIV binding. Quinobene is believed to interfere with the interaction of the viral envelope and the cellular membrane which occurs after viron binding [3]. Because of the potential of quinobene in anti-HIV chemotherapy, a specific and accurate chemical assay for quinobene is needed.

Water soluble sodium salts of organic polysulphonic acids, such as suramin, have been successfully analysed with high-performance liquid chromatography (LC) [4–8]. In those cases, reversed-phase columns were used. Since quinobene is reportedly water soluble [2, 3], reversed-phase LC would appear to be a good analytical technique for quinobene. Because of the ionic nature of quinobene, capillary electrophoresis (CE) would also be suitable for quinobene analysis. Indeed, sodium salts of anthraquinone mono- and di-sulphonic acid isomers have been successfully separated by CE [9, 10]. Therefore, the use of CE for quinobene analysis was also explored. This paper presents the LC and CE assays developed for quinobene, a member of a new class of anti-HIV agents.



Quinobene (S)

Figure 1
Chemical structure of quinobene.

Experimental

Reagents and materials

Quinobene, lots LK-17-17-3, LK-17-24-1, 873.A.91.2 and 873.A.92.301 were received from the US National Cancer Institute. 2-Amino-3,5-diiodobenzoic acid and sulphanilic acid were purchased from Eastman Organic Chemicals (Rochester, NY). 4,4′-Diamino-stilbene-2,2′-disulphonic acid and 8-hydroxy-quinoline-5-sulphonic acid 1-hydrate were purchased from Eastman Kodak (Rochester, NY). Glacial acetic acid was from J.T. Baker (Phillipsburg, NJ). Methanol (LC grade), boric

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acid and tris(hydroxymethyl)amino-methane (Tris) were purchased from Mallinkrodt (Paris, KY). (Ethylenedinitrilo)-tetraacetic acid, disodium salt, dihydrate (EDTA) was from Aldrich (Milwaukee, WI). Polyethylene glycols (PEG) were purchased from Fluka (Ronokonkoma, NY). The chemicals were reagent grade and were used without further purification. Sample and buffer solutions were prepared with distilled water.

High-performance liquid chromatography (LC)

LC was performed on an IBM LC/9533 ternary gradient liquid chromatograph equipped with an IBM LC/9505 automatic sample handler, and a BIO-RAD model 1305 UV detector. Data were collected and processed with a Waters Maxima 820 data station. The LC column was Phenomenex, IB-SIL C_{18}, 5 μ, 4.6 × 250 mm. Mobile phase was a 90 min linear gradient of solvent A to solvent B, at a flow rate of 1.0 ml min^{-1}. Solvent A was CH_3OH–HOAc (0.05 M) (1:9, v/v) and solvent B was CH_3OH–HOAc (0.05 M) (9:1, v/v). The column was conditioned with 15 min of solvent A before injection. Detection was by UV at 330 nm. Quinobene solutions (0.5 mg ml^{-1}) were prepared either in water or in an internal standard solution. The internal standard solution was prepared by dissolving 10 mg 2-amino-3,5-diiodobenzoic acid in 0.2 ml 0.2 N NaOH and diluting to 25.0 ml with distilled water. The injection volume was 50 μl.

Capillary electrophoresis (CE)

CE was performed on a Biofocus 3000 Electrophoresis System using a 36 cm × 50 μm glass capillary coated with covalently bonded linear polymer (both from BIO-RAD, Hercules, CA). Run buffer was Tris-boric acid (pH 8.6; 0.3 M) containing 2 mM EDTA and 4% each of 6K, 12K, 20K and 35K PEG. Loading was done hydrodynamically by pressure at 120 psi.s. The run voltage was 12 kV. The analyte ions migrated from the negative to the positive electrode. Detection (UV at 330 nm) was at the positive electrode. Data were collected and processed by the Biofocus 3000 Integration System (BIO-RAD). Quinobene solutions (0.1 mg ml^{-1}) were prepared in an internal standard solution. The internal standard solution was prepared by dissolving 1 mg sulphanilic acid in 25 ml distilled water.

Results and Discussion

Liquid chromatography

Initial LC using an ODS column and a mobile phase of CH_3OH–potassium phosphate buffer (pH 3; 50 mM) (35:65, v/v) resulted in a chromatogram (Fig. 2) which was not reproducible. From injection to injection, the sep-

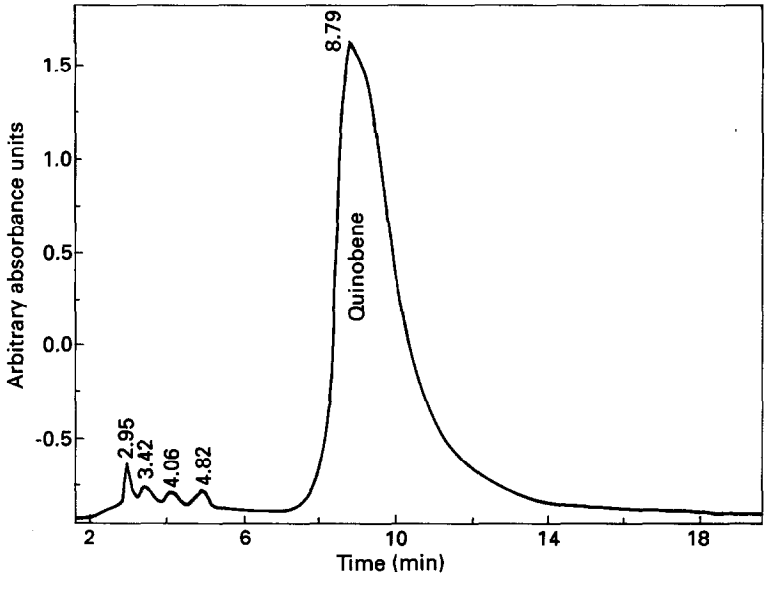

Figure 2
LC chromatogram of quinobene obtained with a C_{18} column (Phenomenex, 5 μ, 4.6 × 250 mm) and a mobile phase of CH_3OH–phosphate buffer (pH 3.0; 20 mM) (35:65, v/v) at 1.0 ml min^{-1}. Detection was by UV at 254 nm.

aration of the peaks were reproducible but the relative intensities of peaks varied significantly. Further investigation indicated that quinobene (the major peak) was partially precipitated out of solution during chromatography, even when only 20 μg was injected. Determination of its solubility revealed that, contrary to the literature [2, 3], quinobene was only sparingly soluble in water (3 mg ml^{-1}). It was soluble in NaOH (0.1 N) but insoluble (<0.01 mg ml^{-1}) in HCl (0.1 N), CH_3OH, CH_3CN or phosphate buffers (pH 3–7; 20–50 mM). Its solubility in HOAc (20 mM) or NH_4OAc (20 mM) was 2–3 mg ml^{-1}, only slightly less than that in H_2O. Due to these solubility limitations, LC development for quinobene was confined to mobile phase of low organic modifier concentration and buffers of weak acid/base. Therefore, normal-phase, ion-pair, and ion-exchange modes of LC were excluded from this assay development. Attempts to benefit from the solubility of quinobene in a base by using an organic polymer-based reversed-phase column (PRP from Hamilton) and high pH (9–12) mobile phase yielded no retention of quinobene. After unsuccessful trials with C_8 and CN columns, the C_{18} column-based gradient LC system described in the Experimental section was developed for quinobene.

Figure 3(a) is a typical chromatogram of quinobene obtained with the developed gradient LC method. The method was specific for quinobene. It resolved quinobene and the internal standard (IS, 2-amino-3,5-diiodobenzoic acid) from the starting materials (4,4′-diaminostilbene-2,2′-disulphonic acid and 8-hydroxyquinoline-5-sulphonic acid) and the photo-transformed products of quinobene [Fig. 3(b) and (c)]. The photo-transformed products were obtained by exposing a quinobene solution (0.5 mg ml^{-1} H_2O) to direct sunlight for 4 h. Under UV irradiation, the *trans* configuration of quinobene along the diazo linkages was inverted to the *cis* configuration [11]. The precision of the method, based on six injections of a single standard solution or single injection of six standard solutions (0.5 mg quinobene plus 0.4 mg IS ml^{-1} H_2O), were 0.6 or 1.0%, respectively. Based on data from the five standard solutions (Table 1), the LC assay was linear ($r = 0.9995$) and accurate (1.1% error). Based on a 3:1 signal-to-noise ratio, the observed limit of detection for quinobene was 0.5 μg.

Although the LC assay was specific, reproducible and accurate, its efficiency was poor. The plate number was <1000 m^{-1}. The quinobene peak was non-symmetrical. Its asymmetry at 10% height of 5 was more than three times those of the starting materials or the internal standard. In addition, while a couple of oversize injections were sufficient to achieve column suitability for the internal standard or the starting materials, at least 10 times the amount was needed for quinobene. Irreversible adsorption onto the column was, apparently, a more serious problem for quinobene than for other sulphonates. Thus, much more conditioning effort would be required before the LC system is suitable for assay.

Capillary electrophoresis

Williams *et al.* [9] have shown the advantage of using CE to analyse anthraquinone sulphonic acids. Anthraquinone mono- and di-sulphonic acid isomers have been separated by CE using a sodium borate buffer (pH 10; 50 mM) and a coated capillary [10]. Since the anionic nature of quinobene is similar to that of anthraquinone sulphonic acids, a similar CE condition, a coated capillary and borate buffer (pH 8.5; 50 mM) was initially attempted to separate the quinobene sample. This resulted in an electropherogram of poorly resolved peaks (Fig. 4). Substituting the borate buffer with Tris-boric acid buffer (pH 8.5; 50 mM) containing 2 mM EDTA did not improve the separation. Zhu *et al.* [12] showed that using a linear polymer such as methylcellulose or PEG as additives in the buffer facilitated CE separation by generating a molecular sieving effect. Although a polyacrylamide gel-filled capillary was recommended for size separation [13], PEG passed through the narrow-bore capillary easier, was more convenient to use, and yielded more reproducible results. Based on this information, 4% each of 6K, 12K, 20K and 35K PEG was added to the Tris-boric acid buffer (pH 8.6; 300 mM) containing 2 mM EDTA. This resulted in a remarkable improvement in the separation of the quinobene sample (Fig. 5). The plate number was in excess of 60,000 m^{-1}, more than 60 times the efficiency of the LC system. The peak was symmetrical. The separation achieved under the CE conditions described in the Experimental section was specific for quinobene. Figure 6 shows that quinobene was resolved from its starting material (4,4′diaminostilbene-

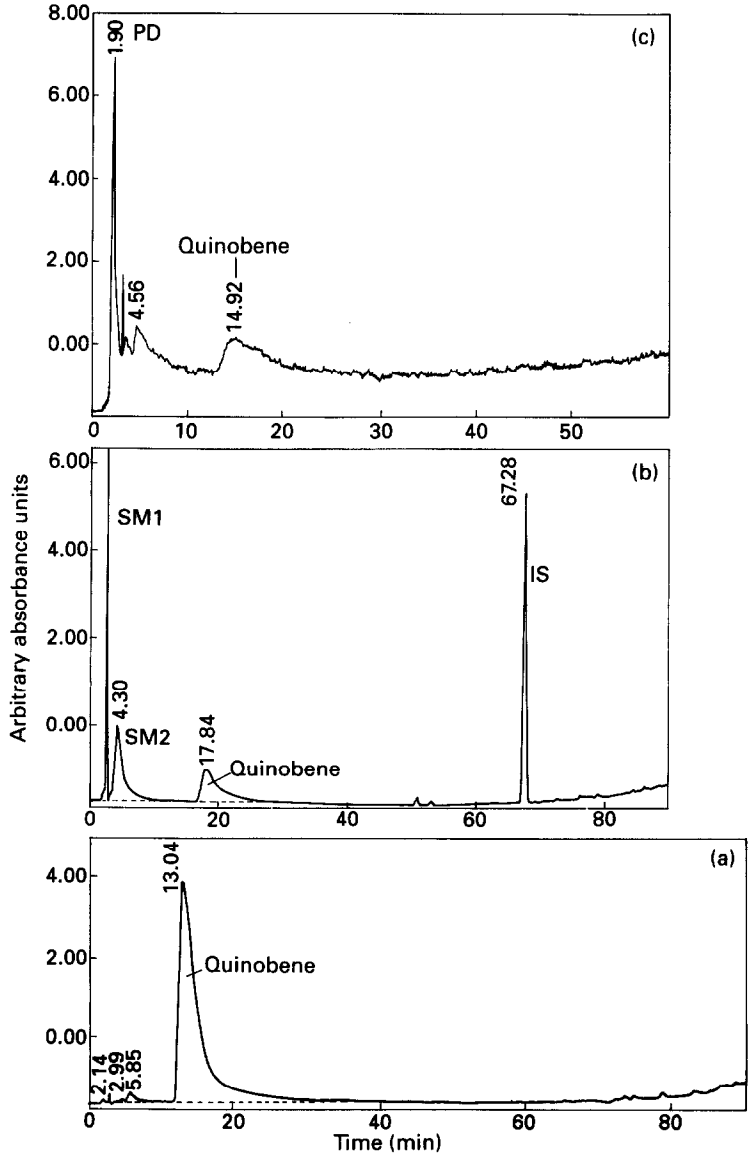

Figure 3
LC chromatograms of the gradient elution (see Experimental section for details). (a) Quinobene in water (0.5 mg ml^{-1}), (b) quinobene plus 2,3-diiodobenzoic acid (IS) in water (0.5 and 0.4 mg, respectively, ml^{-1} H_2O) and starting materials: 4,4′-diaminostilbene-2,2′-disulphonic acid (SM1), 8-hydroxyquionoline-5-sulphonic acid (SM2), and (c) the photo-transformed quinobene products (PD).

Table 1
Linearity and accuracy of LC assay for quinobene (S)

Sample	Peak area			mg ml^{-1} IS solution		
	S	IS	R_A*	Actual	Found†	Error‡ (%)
1	4181	2600	1.608	0.2594	0.2581	0.5
2	6622	2624	2.524	0.3716	0.3799	2.2
3	9208	2595	3.549	0.5254	0.5162	1.7
4	11786	2615	4.507	0.6458	0.6435	0.4
5	13964	2601	5.368	0.7536	0.7580	0.6
					Mean	1.1

See text for LC conditions. Linear regression analysis of R_A (y) vs actual S concentration (x) gave $y = 0.13296x + 0.0443$, $r = 0.9995$.

*Ratio = S/IS.

†Found = $(R_A - 0.0443)/0.13296$.

‡Deviation of found from actual value.

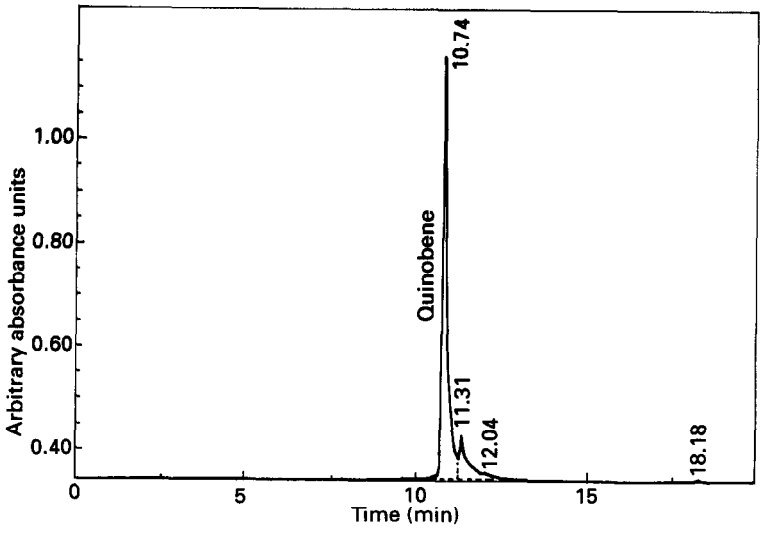

Figure 4
CE electropherogram of quinobene (50 μg ml^{-1} H_2O) obtained with the coated capillary (50 μ i.d. × 36 cm) and borate buffer (pH 8.5; 50 mM).

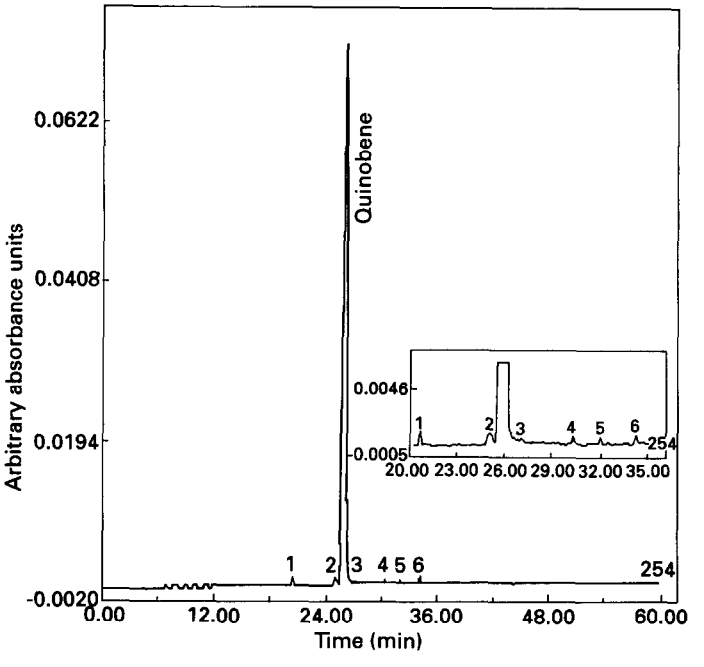

Figure 5
CE electropherogram of quinobene (100 μg ml^{-1} H_2O) obtained with the coated capillary (50 μ i.d. × 36 cm) and Tris-boric acid buffer (pH 8.5; 300 mM) containing 2 mM EDTA and 4% each of 6K, 12K, 20K and 35K PEG. See Experimental section for details.

2,2′-disulphonic acid and 8-hydroxyquinoline-5-sulphonic acid), the photo-transformed *cis* isomers, and the internal standard (sulphanilic acid).

The quantitative aspect of CE analysis required more careful experimental design and is discussed in more detail in a separate paper [14]. To maximize the precision and accuracy of the assay, an automatic instrument with temperature control was used. The sample was loaded hydrodynamically from individual aliquots of sample solution containing the internal standard. The total ionic concentration of the sample solution was less than 1% of that of the buffer. The inner wall of the capillary was coated, washed with water and the capillary

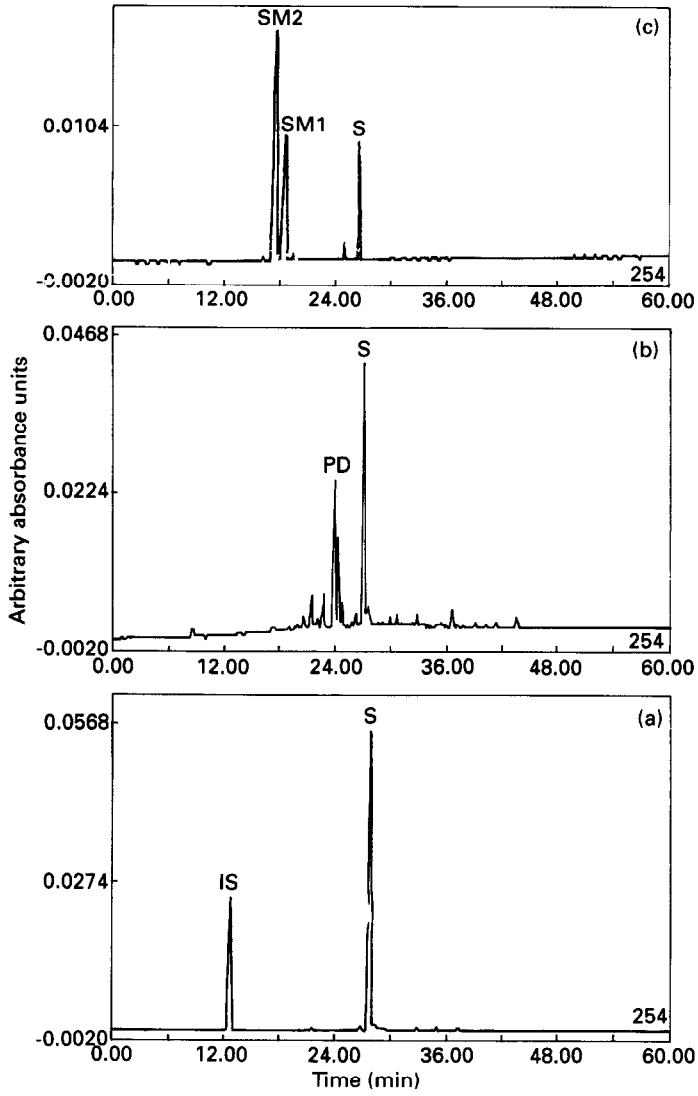

Figure 6
CE electropherograms of (a) quinobene (S) plus sulphanilic acid (IS) in water (100 and 4 μg ml^{-1}, respectively), (b) photo-transformed (4 h under direct sunlight) quinobene solution (100 μg ml^{-1} H_2O) and (c) quinobene plus 4,4′-diaminostilbene-2,2′-disulphonic acid (SM1) and 8-hydroxyquinoline-5-sulphonic acid (SM2). See Experimental section for details.

Table 2
Linearity and accuracy of CE assay for quinobene (S)

Sample	Peak area			μg ml^{-1} IS solution		
	S	IS	R_A*	Actual	Found†	Error‡ (%)
1	1734	1571	1.105	59.1	60.8	2.9
2	2136	1502	1.422	82.1	80.6	1.8
3	2475	1425	1.736	100.3	100.3	0.1
4	3002	1422	2.111	124.7	123.7	0.9
5	3610	1407	2.565	152.6	152.1	0.3
6	4058	1380	2.940	174.2	175.5	0.7
					Mean	1.1

See text for CE conditions. Linear regression analysis of R_A (y) vs actual S concentration (x) gave $y = 0.01601x + 0.131$, $r = 0.9996$.
*Ratio = S/IS.
†Found = $(R_A - 0.131)/0.01601$.
‡Deviation of found from actual value.

Table 3
Comparison of CE and LC assay results

Quinobene lot	LK17-24-1	873.A.91.2	873.A.92.301
CE (7/92)	82.8	80.6	—
CE (8/92)	83.2	80.6	—
CE (9/92)	81.8	79.3	—
CE (11/92)	—	—	87.2
HPLC assay	82.8	77.8	87.4

See text for CE conditions. Results are referenced to quinobene lot LK17-17-3, established by elemental, thermal, spectral and chromatographic data as 80.4% quinobene.

refilled with the buffer before each electrophoresis run. Its i.d. was less than 100 μm. Each test solution was electrophoresed in triplicate. Results were calculated from the peak area ratio of quinobene/IS. Under these conditions, the precision of the CE assay based on six samplings of a single standard solution or single samplings of seven standard solutions (100 μg quinobene plus 40 μg IS ml^{-1} H_2O) was 1.9%. Based on data (Table 2) from six standard solutions (50–175 μg quinobene plus 40 μg IS ml^{-1} H_2O), the assay was linear (r = 0.9996) and accurate (1.3% error). The assay has a linear range of 1–2000 μg of quinobene per ml of IS solution (40 μg IS ml^{-1} H_2O). The limit of quantitation (LOQ) was 1 μg ml^{-1}. The assay was rugged. Day-to-day assay results were consistent and compared well with those of LC assays (Table 3).

Conclusions

A LC and a CE assay have been developed for the quantitation of quinobene, a polysulphonic acid azo dye derivative. Both assays can be adapted for other members of this new class of anti-HIV agents. While both assay are comparable in specificity, precision and accuracy, the CE assay is more rugged, efficient and sensitive than the LC assay.

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