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Note

Simultaneous determination of desferrioxamine and ferrioxamine by high-performance liquid chromatography with amperometric detection

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Desferrioxamine B (DFA), a naturally occurring trihydroxamic acid, is an important drug in the treatment of certain iron overload disorders, such as Cooley's anaemia, and for dialysis encephalopathy (aluminium poisoning) in patients undergoing prolonged dialysis [1]. As a result, interest has grown over the last few years in the determination of DFA using various analytical techniques. Romero and Day [2] used differential pulse polarography at a static mercury drop electrode to determine DFA in dialysate samples by polarographic titration with Fe^{III}. Allain et al. [3] determined DFA, its 1:1 complex with Fe^{III}, ferrioxamine (FA), and the aluminium complex of DFA, aluminoxamine (AlA), by an indirect micro-scale method. FA, AlA and DFA (after complexation with Fe^{III}) were extracted from blood plasma using benzyl alcohol and then determined by atomic absorption spectroscopy with electrothermal atomisation. Inductively coupled plasma atomic emission spectrometry has been used by Bourdon et al [4]. This method again uses the addition of Fe^{III} to DFA, followed by extraction using benzyl alcohol. The sample is then back-extracted into acid for analysis.

In recent years there have been several publications on the use of reversed-phase high-performance liquid chromatography (HPLC) in the analysis of DFA and FA. Cramer et al. [5] reported the determination of DFA by HPLC on octadecylsilica utilising the absorptivities of both DFA and FA at 225 nm

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and of FA at 435 nm. However, the work suffered from interferences from the presence of Fe^{III} on the column and the formation of FA after the column necessitating the addition of EDTA to the mobile phase. Kruck and Kalow [6] were able to quantify FA and study the pharmacokinetics of DFA in blood without the incorporation of EDTA in the mobile phase, using a stainless-steel system. Hughes et al. [7] used a similar system for the estimation of FA and AlA in plasma, using C_{18} solid-phase extraction. Van der Horst et al. [8] investigated direct serum injection using a pre-column, following centrifugation. Recently, Venkataram and Rahman [9] used a metal-free HPLC system to quantify both DFA and FA without a chelating agent in the mobile phase.

The electrochemical oxidation undergone by hydroxamic acids has been studied recently by a number of workers [10–12]. Our laboratory has just reported the use of HPLC with amperometric detection in the analysis of a series of dihydroxamic acids [13]. This paper will describe the use of HPLC coupled with amperometric detection for the analysis of a trihydroxamic acid. DFA and FA can be determined simultaneously, with amperometric detection, yielding the lowest published limit of detection for DFA.

EXPERIMENTAL

HPLC apparatus

The HPLC system was metal-free and consisted of a Dionex analytical pump and an inert Dionex high-pressure injection valve with a commercially packed Dionex RPIC C_{18} (particle size 10 μm) analytical column (25 cm$\times$6 mm I.D.$\times$10 mm O.D.). The UV–visible detector used was a Waters Lambda-Max Model 481 variable-wavelength spectrophotometer. The amperometric detector was a (TL-5A) flow cell from Bioanalytical Systems (West Lafayette, IN, U.S.A.), the electrode cell being of the thin-film design. The working electrode was glassy carbon with a platinum auxiliary and Ag/AgCl reference electrode. A Metrohm 626 Polarecord was used to apply a d.c. waveform and two Philips 8251 strip-chart recorders plotted the detector outputs. The injection volume was 10 μl at a flow rate of 1.3 ml/min. The static cell analysis of the mobile phases was carried out using an EG&G Model 174A polarographic analyser to apply a d.c. waveform and an Omnigraphic 100 recorder. The working electrode was glassy carbon, the auxiliary platinum and the reference a saturated calomel.

Mobile phase composition

Reagents for mobile phase preparation were of AnalaR grade and all mobile phases used were filtered and degassed on a Millipore HPLC filtration system using 0.45-μm filters. Unless otherwise stated the organic modifier was held constant at 33% (v/v) methanol and $1 \cdot 10^{-3}$ *M* sodium chloride was incorporated as a background electrolyte. The control of pH was maintained with

$1.5 \cdot 10^{-2}$ *M* sodium acetate buffer (pH 4.7) and a number of compositions were prepared with varying added concentrations of either EDTA or citrate. The final selected mobile phases used $1 \cdot 10^{-3}$ *M* EDTA or $5 \cdot 10^{-3}$ *M* citrate.

Preparation of standard solutions

Standard stock solutions of 0.02 *M* desferal mesylate (Ciba Geigy, Horsham, U.K.) and 0.1 *M* AnalaR ferric nitrate nonahydrate were prepared in triply distilled water. The iron solution contained 1% (v/v) nitric acid. Standard solutions of desferal were then made by appropriate dilution (range $1 \cdot 10^{-3}$ to $2 \cdot 10^{-8}$ *M*), while ferrioxamine was prepared by addition of equimolar amounts of desferal and Fe^{III} solution, followed by dilution (range $1 \cdot 10^{-3}$ to $1 \cdot 10^{-7}$ *M*). Mixtures were prepared by adding a known excess of DFA.

Preparation of spiked plasma samples

Freeze-dried human blood plasma was obtained from the Irish Blood Transfusion Service. This was reconstituted as instructed by the addition of 400 ml of distilled water supplied with the plasma, portions then being taken and spiked with DFA and FA. A stock solution containing 0.025 *M* FA and 0.05 *M* DFA was used from which microlitre quantities were added to 5 ml of plasma to give concentration ranges of $5 \cdot 10^{-5}$ to $5 \cdot 10^{-4}$ *M* FA and $1 \cdot 10^{-4}$ to $1 \cdot 10^{-3}$ *M* DFA.

Pre-treatment of plasma samples

The following procedure was chosen as it provided the maximum sample clean-up. A Sep-Pak C_{18} cartridge (Waters Assoc.) was first wetted with 10 ml of methanol, followed by 10 ml of $1 \cdot 10^{-2}$ *M* 1-octanesulphonic acid (OSA) (Sigma) in methanol and 20 ml of $1 \cdot 10^{-2}$ *M* OSA in water. Samples (2 ml) were then applied to the cartridge, washed with 10 ml of OSA in water and eluted with 5 ml of methanol.

RESULTS AND DISCUSSION

The possibility of directly and simultaneously determining DFA and FA on a metal-free system, without the need to incorporate a chelating agent in the mobile phase, was investigated with UV detection at 225 nm. The system was purged before analysis with 20 m*M* DFA, as discussed by Cramer et al. [5]. This, however, only proved to be a short-term cure as evidence of complex formation on injection of DFA soon re-appeared. Fig. 1 shows an injection of DFA into a mobile phase of acetate–methanol with clear evidence of a reaction zone before the DFA peak. This is in contrast to the results reported by Venkataram and Rahman [9]. As a result, an iron-chelating reagent was incorporated into the mobile phase and the effect on the chromatographic behaviour and detector response recorded. If the EDTA in the mobile phase is increased

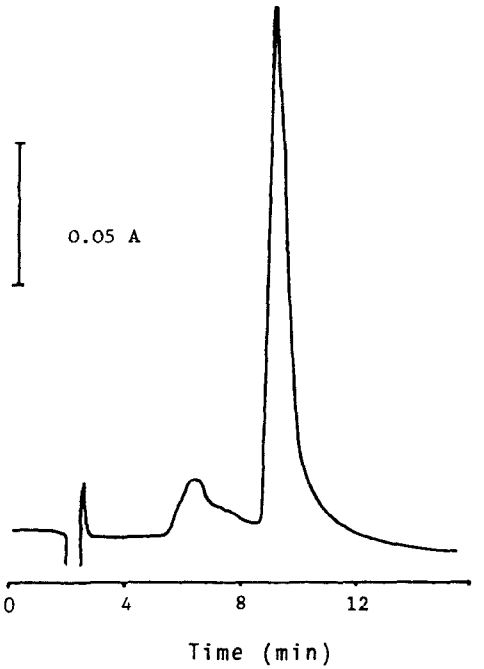

Fig. 1. Injection of DFA into a metal-free system. Mobile phase, 33% (v/v) methanol in $1.5 \cdot 10^{-2}$ *M* acetate buffer (pH 4.7); injection volume, 10 µl; flow-rate, 1.3 ml/min. Detection at 225 nm. $[DFA] = 2 \cdot 10^{-3}$ *M*.

progressively towards $1 \cdot 10^{-3}$ *M* the FA peak is seen to slowly decrease in size until no FA is detected at all.

Incorporation of EDTA in the mobile phase is ideal to suppress complex formation when UV–visible detection is being used. The selection of the applied potential for the amperometric detection of DFA and FA followed from studies of the voltammetry of the analytes in the flowing system. Fig. 2 shows the effect of variation of the applied potential on the electrochemical responses. A value of +1.1 V was chosen for simultaneous detection of DFA and FA. However, the use of EDTA for chromatographic analysis results in a much reduced amperometric response when compared to the response when EDTA is omitted. Voltammograms of the mobile phases recorded in a static cell explain the high background current as being due to the electrochemical activity of EDTA at the applied potential, +1.1 V (versus Ag/AgCl) (Fig. 3). The need for another chelating agent was obvious, one which would suppress complex formation on-column, not remove Fe^{III} from FA and be electrochemically inactive at +1.1 V.

Citric acid was considered a possible substitute for EDTA based on its stability constant for Fe^{III} complexation and on its voltammetric response in Fig.

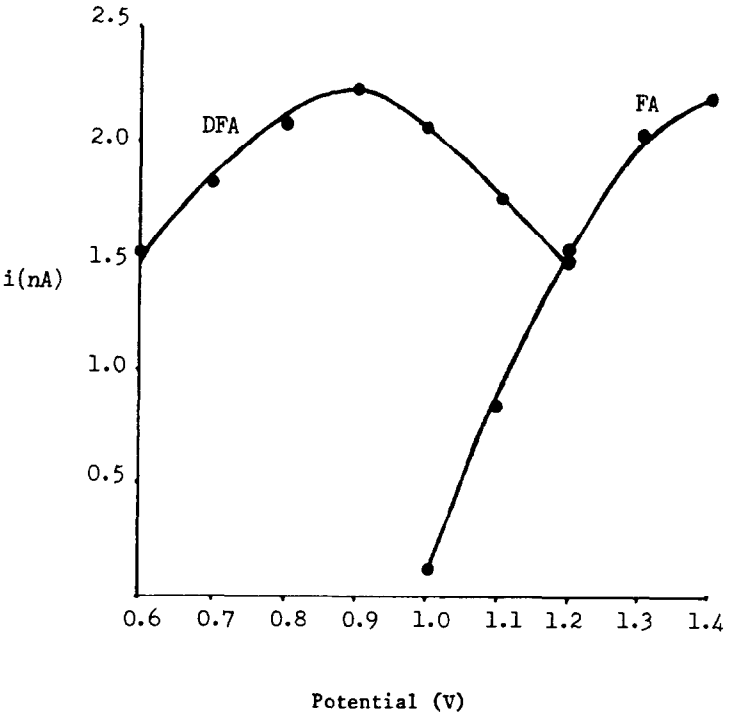

Fig. 2. Electrochemical response as a function of potential (versus Ag/AgCl) for DFA and FA at pH 4.7. [DFA] = $1.1 \cdot 10^{-2}$ *M*; [FA] = $1.0 \cdot 10^{-2}$ *M*.

3 (log K_1 for Fe^{III}–EDTA is 25.0 compared to 11.2 for Fe^{III}–citrate [14]). A mobile phase with $5 \cdot 10^{-3}$ *M* citrate satisfied all the criteria and was used throughout the rest of the work. The capacity factors were 2.1 and 4.1 for FA and DFA, respectively.

Linearity and precision

The amperometric and UV detector responses were studied in the ranges $1 \cdot 10^{-5}$ to $1 \cdot 10^{-4}$ *M* DFA and $5 \cdot 10^{-6}$ to $4 \cdot 10^{-5}$ *M* FA, each standard being injected five times. UV detection yielded a linear regression factor for DFA of 0.9997 and for FA of 0.9998. Amperometric detection gave values of 0.9985 for DFA and 0.9943 for FA. Tables I and II summarise a statistical evaluation of the results. In general UV detection is regarded as being more precise. The mean C.V. values were taken as a measure of the precision of the methods with intra-assay values of 6.7 and 3.4% for DFA and FA, respectively, for UV detection and 3.6 and 4.8% with amperometric detection. The tabulated differences between the mean values of replicate assays of the standards and the true values are a measure of the accuracy of the method.

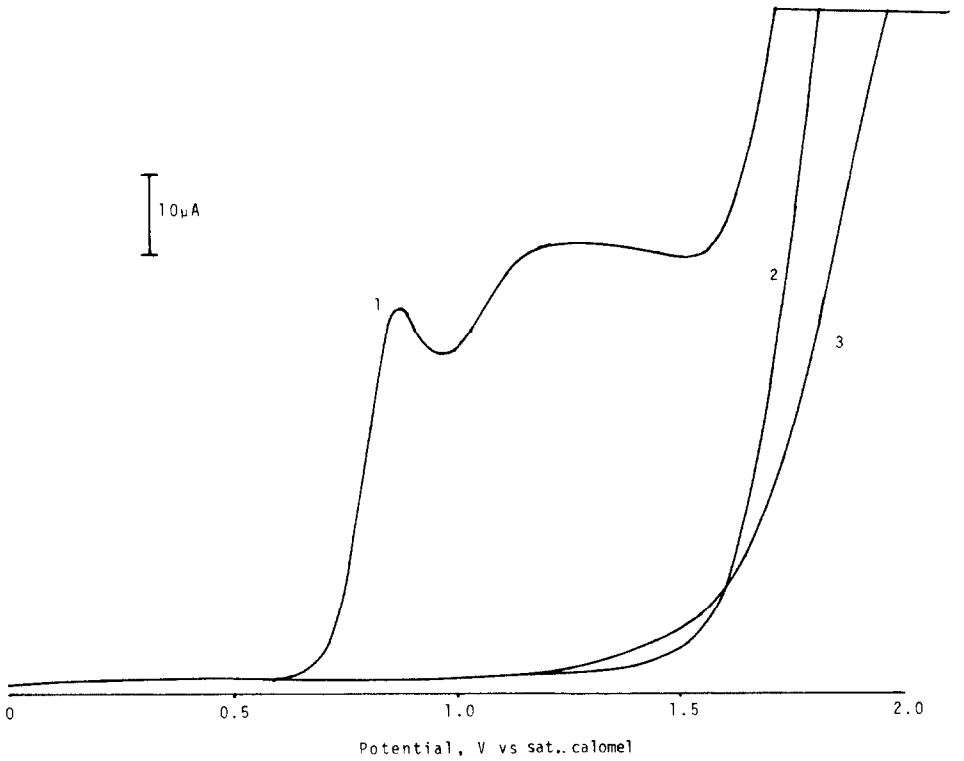

Fig. 3. Voltammogram of different mobile phases: (1) EDTA ($2 \cdot 10^{-3}$ *M*) in $1.5 \cdot 10^{-2}$ *M* acetate buffer (pH 4.7); (2) $1.5 \cdot 10^{-2}$ *M* acetate buffer (pH 4.7); (3) citric acid ($5 \cdot 10^{-3}$ *M*) in $1.5 \cdot 10^{-2}$ *M* acetate buffer (pH 4.7). All mobile phases contain 33% (v/v) methanol.

TABLE I

INTRA-ASSAY PRECISION OF THE HPLC ASSAY OF DFA USING UV AND AMPEROMETRIC DETECTION

Concentration added (10^{-5} *M*)	UV		Amperometric	
	Concentration found (mean ± S.D.) (10^{-5} *M*)	C.V. (%)	Concentration found (mean ± S.D.) (10^{-5} *M*)	C.V. (%)
1	1.2 ± 0.1	8.3	0.8 ± 0.03	3.7
3	3.0 ± 0.1	3.3	3.3 ± 0.20	6.1
5	4.7 ± 0.2	4.2	4.8 ± 0.10	2.1
8	8.2 ± 1.2	1.2	7.9 ± 0.20	2.5

TABLE II

INTRA-ASSAY PRECISION OF THE HPLC ASSAY OF FA USING UV AND AMPEROMETRIC DETECTION

UV			Amperometric		
Concentration added (10^{-6} *M*)	Concentration found (mean ± S.D.) (10^{-6} *M*)	C.V. (%)	Concentration added (10^{-6} *M*)	Concentration found (mean ± S.D.) (10^{-6} *M*)	C.V. (%)
5	4.9 ± 0.2	4.1	8	8.3 ± 0.8	9.6
8	7.9 ± 0.3	3.8	10	10.5 ± 0.4	3.8
10	10.0 ± 0.3	3.0	13	12.4 ± 0.6	4.8
13	13.0 ± 0.4	3.1	18	18.5 ± 0.4	2.1
18	18.0 ± 0.5	2.8	30	28.7 ± 1.5	5.2
			40	41.2 ± 1.3	3.1

Spiked plasma samples

Ion-pairing reversed-phase solid-phase extraction using OSA was employed on the standard DFA and FA solutions and on plasma samples spiked with DFA and FA. The pretreatment method exploits the free protonated amino group of the trihydroxamate and its Fe^{III} complex. Recoveries for standards and plasma samples were very similar at mean recovery values of 67% for DFA and 79% for FA and good linearity was maintained. Typical chromatograms obtained for the plasma samples with UV and amperometric detection are shown in Fig. 4. Noticable features are the high selectivities obtained by both methods, with UV detection less influenced by endogenous peaks. The somewhat poorer performance by amperometric detection is no doubt due to the choice of the applied potential, designed to enhance the response of FA relative to DFA.

Limits of detection

A comparison of the limits of detection for UV, visible and amperometric detection is given in Table III. The limit of detection (signal-to-noise ratio = 2.0) was calculated from a series of measurements made at concentrations of DFA and FA close to the blank level. With constant purging of the mobile phase with nitrogen, it is also possible to quantify the levels of FA by following its reduction at −0.75 V. The limit of detection determined using amperometric detection at this potential is included for comparison. The values obtained using UV detection are comparable with previously reported values [5–7]. In terms of absolute limit of detection [8], a value of 25 ng reported for DFA using on-line preconcentration with UV detection can be compared with a value of 2.6 ng by amperometric detection.

In conclusion, by careful selection of the mobile phase composition and the

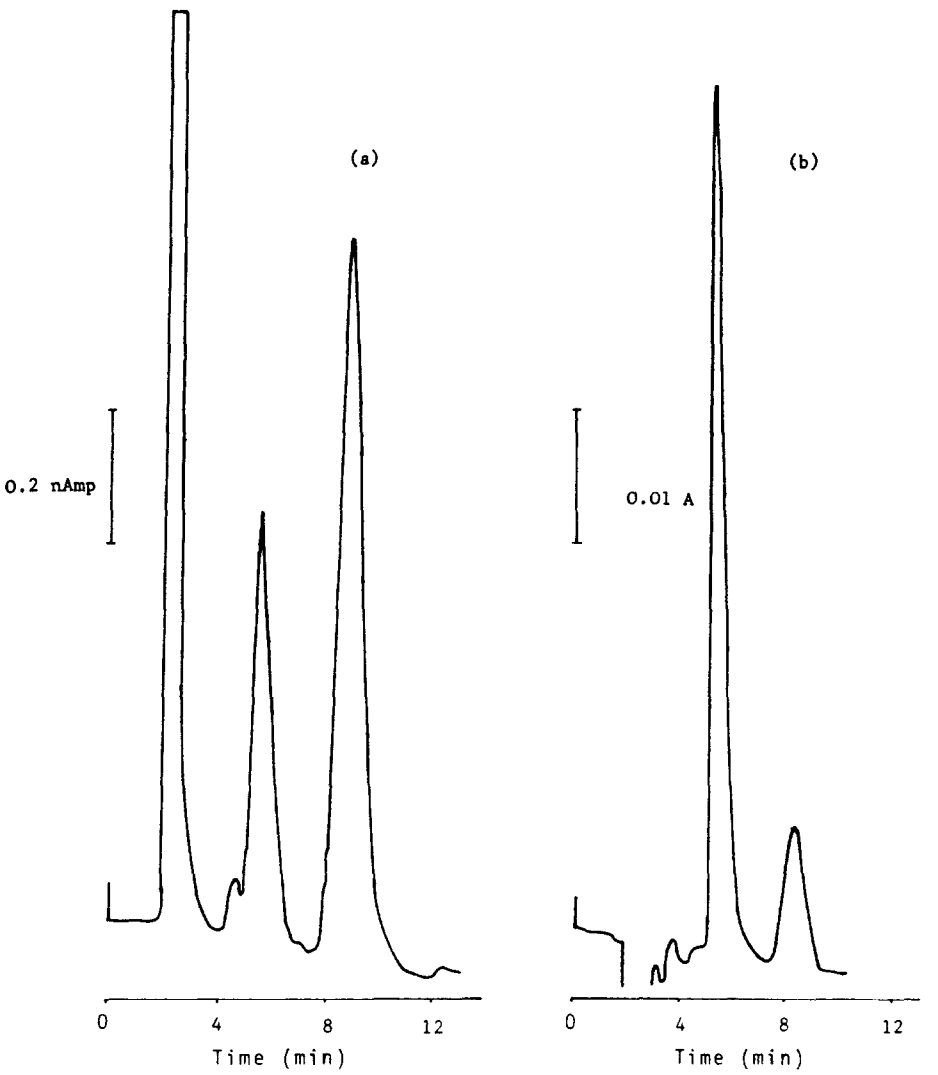

Fig. 4. Typical chromatograms for spiked plasma samples. (a) Amperometric detection at +1.1 V; (b) UV detection at 225 nm. Retention times: DFA, 8.6 min; FA, 5.4 min. [DFA] = $3 \cdot 10^{-4}$ *M*; [FA] = $2 \cdot 10^{-4}$ *M*.

TABLE III

LIMITS OF DETECTION (LOD) WITH UV-VISIBLE AND AMPEROMETRIC DETECTION

UV-visible		Amperometric	
Analyte	LOD (*M*)	Analyte	LOD (*M*)
DFA (225 nm)	$1.83 \cdot 10^{-6}$	DFA (+1.1 V)	$4.12 \cdot 10^{-8}$
FA (435 nm)	$2.31 \cdot 10^{-5}$	FA (+1.1 V)	$8.36 \cdot 10^{-7}$
FA (225 nm)	$5.78 \cdot 10^{-7}$	FA (−0.75 V)	$5.80 \cdot 10^{-6}$

applied potential, DFA and FA can be directly and simultaneously determined by HPLC with amperometric detection. The method is applied to spiked plasma samples and a comparison of UV, visible and amperometric detection shows that amperometric detection yields the lowest limit of detection. Further work on the application of the method in the clinical analysis of DFA and new chelation therapy drugs would be valuable.

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