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INDIRECT DETECTION OF INORGANIC ANIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: USE OF PAPAVERALDINIUM AS AN ULTRAVIOLET ABSORBING AGENT

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SUMMARY

An indirect UV detection method, using tetrabutylammonium hydroxide and papaveraldine perchlorate as counter ion and ion interaction reagent, respectively, is described. Water analysis and drug monitoring in the treatment of childhood epilepsy with bromides are presented as possible applications. As a result of analytical difficulties, a so-called "overload effect" was studied and a theoretical mechanism for this is proposed.

INTRODUCTION

Ion chromatography was first used to analyse ionic species[1]. However, this technique has the drawbacks of the fragility and cost of the column and the need for a thermostatic system. In addition, in the Dionex system, the suppressor column has to be regenerated after a few hours of use. Although a very low limit of detection can be reached, ion chromatography needs special apparatus and careful maintenance.

Indirect detection has been used for about 10 years. This simple technique allows the detection and quantification of compounds that are outside the scope of other detection techniques[1–3]. An ultraviolet-absorbing ion interaction reagent (IIR) is added to the mobile phase and then distributed to the stationary phase. This agent forms ion pairs with solutes of opposite charge which are eluted and detected easily through absorbance of the IIR[4]. The detection mechanism for this kind of chromatography is complex and has already been described by Stranahan and Deming[5,6].

In this study, ion-pair chromatography with indirect detection was chosen because with this method it is possible to assay inorganic anions with conventional apparatus. C_{18} columns were used because their efficiency and mechanical behaviour are better than those of ion-exchange resins. Many parameters (mobile phase

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composition, solvent concentration, pH, ionic strength) can be modified when a C_{18} stationary phase is used. Such flexibility allows better optimization of the chromatographic conditions while setting up the assay. Further, the use of each column over an extended period is possible because of the stability of the stationary phase. Two major applications were considered: first, water analysis needs a fast, automatable technique in order to assay several anions simultaneously, and second, this technique allows the assay of blood bromide. Analytical difficulties appeared in the quantification of anions and in the choice of an internal standard, wich led us to study a so-called "overload effect".

EXPERIMENTAL

Reagents

Papaveraldine was synthesized from papaverine[7,8]. The perchlorate salt was obtained by dissolving 353 mg of papaveraldine base in 20 ml of 0.7% (w/v) perchloric acid, diluting to 100 ml with distilled water and heating at 90°C until dissolution was complete. Crystallization occurred after filtration within 24 h at 4°C (90% yield).

Tetrabutylammonium hydroxide (TBAH) [40% (w/v) aqueous solution] was obtained from Sigma, (Paris, France), acetonitrile (LiChrosolv, for chromatography) from Merck (Paris, France), mineral anions and citric acid (Normapur quality) from Prolabo (Paris, France) and water [high-performance liquid chromatographic (HPLC) grade] from FSA (Loughborough, U.K.).

Preparation of the mobile phase

A 50-mg amount of papaveraldine perchlorate was added to 850 ml of $7 \cdot 10^{-3}$ *M* sodium citrate buffer (pH 3.2) and dissolved by sonication, then 1.75 g of TBAH were added and the solution was mixed with 150 ml of acetonitrile. The resulting mobile phase was filtered on a 0.22-μm filter (GWMP; Millipore, France). The final pH was about 3.5.

Chromatographic procedures

The column was first washed with acetonitrile–water (50:50, v/v), then loaded with the mobile phase for 1 h in order to achieve adsorption of TBAH and papaveraldine on the C_{18} stationary phase. The mobile phase used during this operation was discarded. The system was then recycled and equilibrated for 24 h.

Apparatus

A Model 850 modular system from DuPont (Paris, France) was used, consisting of an HPLC pump, a thermostatic module set at 35°C and a variable-wavelength detector set at 325 nm. Peak areas and peak heights were determined with a Shimadzu Model CR5A integrator (Touzard et Matignon, France). The column used was a 10-μm C_{18} μBondapak (250 × 4.6 mm I.D. from Waters (Paris, France).

Calculations

The number of theoretical plates, N, was calculated using the equation $N = 5.54(t_R/\delta)^2$, where δ is the peak width at half-height and t_R is the retention time[9].

The displacement ratio (DR) was calculated from[10,11]

$$DR = \frac{\text{papaveraldine peak concentration}}{C_{max}}$$

with (from ref. 9):

$$C_{max} = \frac{Q_{inj}}{V_R}\sqrt{\frac{N}{2\pi}} \tag{13}$$

where Q_{inj} is the amount of anion injected, V_R the retention volume and the papaveraldine peak concentration = peak absorbance/εl (where ε = molar absorbance coefficient and l = length of the detector cell).

Study of overload effect

Response factors for internal standards and anions have to be strictly independent. In order to study this property, we examined the linearity and parameters of calibration graphs for NaCl with or without added $NaNO_3$. Four calibration graphs were prepared in $7 \cdot 10^{-3}$ *M* citrate buffer: (A) $5 \cdot 10^{-4}$–$50 \cdot 10^{-4}$ *M* NaCl solution; (B) $5 \cdot 10^{-4}$–$50 \cdot 10^{-4}$ *M* NaCl solution containing 10^{-2} *M* $NaNO_3$; (A′) $5 \cdot 10^{-5}$–$1 \cdot 10^{-3}$ *M* NaCl solution; and (B′) $5 \cdot 10^{-5}$–$1 \cdot 10^{-3}$ *M* NaCl solution 10^{-3} *M* $NaNO_3$. The different sample concentrations represented by A, B, A′ and B′ were injected alternately to minimize the time-dependent variability of the elution equilibrium. Two further solutions of 10^{-2} and 10^{-3} *M* $NaNO_3$ were injected alternately with the above solutions in order to study the response to nitrate alone and in the presence of chloride.

Procedures used for blood bromide assay

The mobile phase was similar to that described above with papaverinium fluoride as the detection agent because it is more soluble than perchlorate: 10^{-4} *M* papaverinium fluoride was added to 900 ml of $7 \cdot 10^{-3}$ *M* sodium citrate buffer, then $2.5 \cdot 10^{-3}$ *M* TBAH added and the solution was mixed with 100 ml of acetonitrile. The extraction of bromide from blood (98% yield) was performed on whole blood: 1 ml of methanol was added to 500 µl of blood, shaken for 30 s and centrifuged at 1000 *g* for 10 min. A 100-µl volume of the supernatant was diluted with 2 ml of distilled water and 20 µl of the mixture were injected. Cl^- and Br^- anions were identified by standard additions to the samples.

RESULTS AND DISCUSSION

Effects of the solvent

The chromatograms show successively a first peak corresponding to the void volume, either positive or negative depending on the composition of the injection solvent; a double system peak, first negative, then positive, the size of which depends on the composition of the injected sample; a positive peak, wich may be used to detect the injected anion, corresponding to the desorption of the excess IIR previously adsorbed during the elution of the anion along the column, and a final negative peak corresponding to the retention time of the perchlorate anion.

TABLE I

RETENTION CHARACTERISTICS LIMIT OF DETECTION (SIGNAL-TO-NOISE RATIO = 3) AND LIMITING CONCENTRATIONS

Parameter	$H_2PO_4^-$	NO_2^-	Cl^-	Br^-	NO_3^-	I^-	SO_4^{2-}
t_R (min)	5.5	5.8	6.5	7.1	7.6	9.5	18.9
k'	2.06	2.22	2.61	2.94	3.22	4.28	9.50
N (plates/m)	14 000	15 500	19 500	23 200	24 200	6700	18 300
L.O.D. (mg/l)	9.5	2.7	1.4	3.2	2.5	4.8	3.8
European Pharmacopoeia (mg/l)	5	0.005	50	–	10	–	50

Retention characteristics

Table I and Fig. 1 give data and chromatograms for the seven anions studied in aqueous solution. The efficiency of the separation is about 20 000 theoretical plates per metre. This system is therefore among those with the best performance. The linearity of the method was studied over a range of 50 nmol injected. The calibration graph (peak height *versus* amount injected) shows good linearity from 1 to 25 nmol injected. Good and easy quantification was obtained from measurements of peak heights.

Within-day reproducibility

Successive injections of various concentrations of NaCl gave satisfactory results for peak-height variability (Table II).

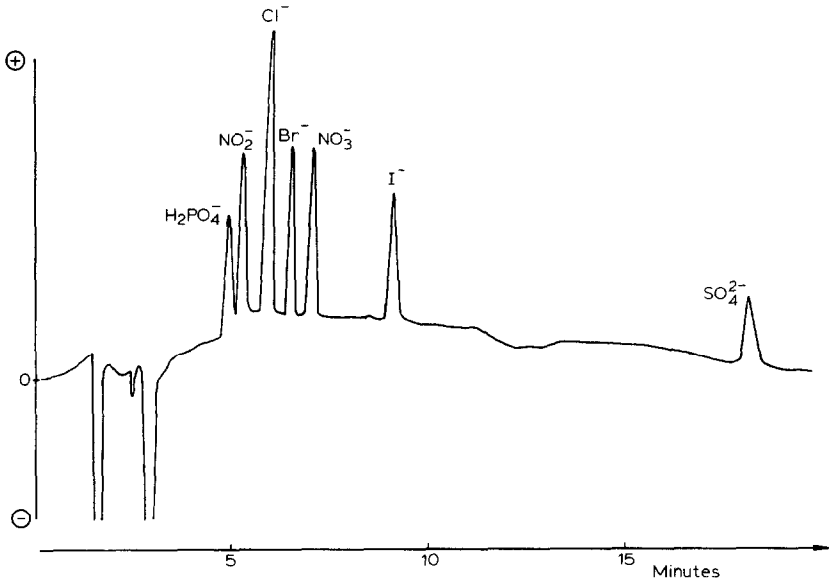

Fig. 1. Separation of phosphate, nitrite, chloride, bromide, nitrate, iodide and sulphate anions. For conditions, see text.

TABLE II
WITHIN DAY REPRODUCIBILITY

Chloride sample concentration (M)	*Coefficient of variation (%) (n=7)*
$2 \cdot 10^{-4}$	8.06
$5 \cdot 10^{-4}$	2.76
10^{-3}	3.9
$4 \cdot 10^{-3}$	2.68

Choice of the retention counter-ion

Two counter ions (tetrabutylammonium hydroxide and a detection agent) were adsorbed on the C_{18} stationary phase. Both retain the injected anions by electrostatic interactions. TBAH, called the retention counter ion, is the more effective retention agent. It was present at a concentration twenty times higher than that of the detection agent. Its adsorption to the stationary phase is easily reversible, in contrast to the long-chain alkylammonium[1]. It is important to use the hydroxide form rather than another salt, as this is eluted less readily.

A comparison of the effect of various concentrations on the retention efficiency showed that a steady state is reached at about 3.0 μM (Table III).

TBAH has no absorbance at the wavelength used for detection.

Choice of papaveraldine perchlorate

This detection agent gives a strong background signal at 325 nm and permits the indirect detection of anions. In order to obtain a high enough solubility of papaveraldine, a salt form was used. We chose the perchlorate, which did not interfere with the anions tested. The weak solubility of the salt meant that the maximum possible concentration of the detection agent was $1.1 \cdot 10^{-4}$ M. This concentration gives an absorbance of about 1, which offers an optimum dynamic reserve[10,11]. However, the retention of anions is insufficient at this concentration, and another counter ion is required (TBAH).

Choice of detection wavelength and limit of detection

Studying the signal-to noise ratio across the UV spectrum we found that the limit of detection (L.O.D.) was constant and maximal corresponding to an absorbance of 0.6–1.2 throughout the wavelength range 200–360 nm (Table IV). The detection

TABLE III
CHOICE OF THE RETENTION COUNTER-ION CONCENTRATION
Mobile phase: 10^{-2} M citrate buffer–acetonitrile (80:20, v/v), containing 10^{-4} M papaveraldine perchlorate and TBAH. Sample KBr concentration: 10^{-3} M.

TBAH (mM)	*t_R Br^- (min)*
2.6	11
2.9	12
3.2	12.5
4.5	12.5

TABLE IV
CHOICE OF THE DETECTION WAVELENGTH

Wavelength (nm)	*Absorbance (a.u.f.s.)*	*Peak height*	*Noise*	*Signal-to-noise ratio*	*L.O.D. (10^{-5} M)*
365	0.6	776	1	776	4
362	0.8	792	1	792	4
354	1.06	896	2	448	7
350	1.24	880	3	293	10

wavelength chosen, *i.e.*, 325 nm, which is an absorbance maximum, gave good reproducibility, owing to the minimal variability in absorbance. Table I gives the L.O.D.s for the anions studied.

Proportion of eluent modifier

A certain amount of acetonitrile (15% minimum) is necessary in order to dissolve the papaveraldine. Increasing this concentration decreases the retention of the anions studied. The lack of solubility of papaveraldine perchlorate in methanol prevents its substitution for acetonitrile.

Composition of the buffer and pH of the eluent

The pH of the eluent had to be around 3 in order to maintain the papaveraldine in an ionized state ($pK_a = 5$). Therefore, citrate buffer ($pK_a = 3.13$) was chosen. An inorganic buffer, interfering with the separation, had to be avoided. The pH has a major influence on the retention and detection of weak acids, for example $H_2PO_4^-$ and NO_2^- (pK_a 2.15 and 3.35, respectively). Increasing the pH of the eluent increases their retention and response factors because of the greater ionization.

Furthermore, an increase in the buffer concentration, *i.e.*, in the ionic strength, decreases the retention of all anions. The buffer adopted allows good resolution of the seven anions tested.

Overload effect

Table V gives the peak areas for Cl^- in the four solutions A, B, A′ and B′. The

TABLE V
OVERLOAD EFFECT

Solution	*Area of chloride peak*								
	NaCl concentration (M)								
	$5 \cdot 10^{-4}$	$10 \cdot 10^{-4}$	$20 \cdot 10^{-4}$	$50 \cdot 10^{-4}$	$5 \cdot 10^{-5}$	$10 \cdot 10^{-5}$	$20 \cdot 10^{-5}$	$50 \cdot 10^{-5}$	$100 \cdot 10^{-5}$
A	73	130	246	590					
B	55	101	193	468					
Ratio B/A	0.753	0.777	0.785	0.793					
A′					354	673	1310	3230	6420
B′					340	646	1250	3076	6110
Ratio B′/A′					0.927	0.943	0.946	0.952	0.953

TABLE VI
OVERLOAD EFFECT: *t*-TEST ON CALIBRATION GRAPHS FOR SOLUTIONS A, B, A′ AND B′

Calibration graph	*Slope*	*Intercept*	*r*	*t-test*
A	11.33	$1.5 \cdot 10^{-3}$	0.999	$t = 16.55$ Degree of freedom = 3
B	9.04	$9.6 \cdot 10^{-4}$	0.999	Slopes significantly different.
A′	63.66	$3.4 \cdot 10^{-4}$	0.998	$t = 4.4$ Degree of freedom = 3
B′	60.53	$3.8 \cdot 10^{-4}$	0.999	Slopes alike

peak areas were lowered on addition of NO_3^- but only with 10^{-2} *M* NO_3^- (solutions A and B). This is characteristic of an overload effect. This was demonstrated (i) by the decrase in the slope (*a*) of Cl^- calibration with 10^{-2} *M* NO_3^- ($a = 11.3$ and 9.04, for solutions A and B, respectively) (see test in Table VI and Figs. 2 and 3); (ii) by the calculation of the ratio *R*:

$$R = \frac{X}{Y} = \frac{Cl^- \text{ area}/NO_3^- \text{ area}}{Cl^- \text{ concentrations}/NO_3^- \text{ concentration}}$$

which theoretically should be constant, but whereas this is true for low Cl^- concentration with 10^{-3} *M* NO_3^- (Table VII), *R* decreases and is more variable with higher anion concentrations (Table VII); and (iii) conversely, the peak area obtained for 10^{-2} *M* NO_3^- alone is higher than in the presence of chlorides.

The overload effect demonstrates the influence of the environment on response factors[12]. The comparison of the *DR* of NaCl solutions with and without nitrate shows a decrease in *DR* when 10^{-2} *M* NO_3^- is present (Table VIII). Further, it is noteworthy

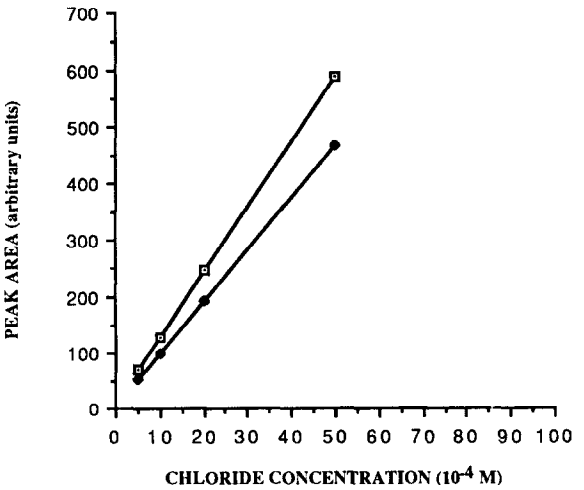

Fig. 2. Calibration graphs for solutions (□) A and (●) B.

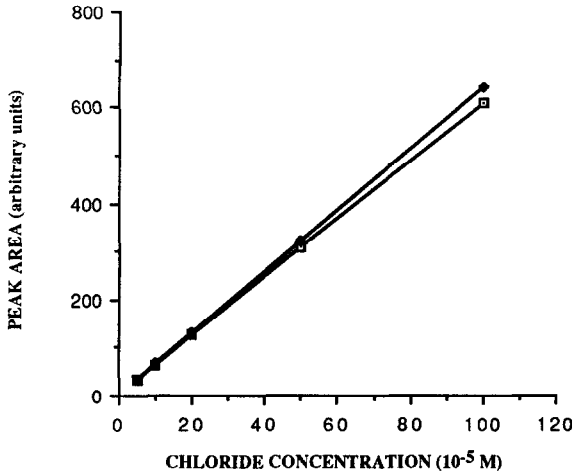

Fig. 3. Calibration graphs for solutions (●) A' and (□) B'.

that the average value of *DR* is about 10^{-2}. The injection of a sample anion at a concentration of 10^{-2} *M* gives rise to ion pairs at a concentration of $10^{-2} \times 10^{-2} = 10^{-4}$ *M*, which is precisely the concentration of the detection agent. Hence all papaveraldine present at the injection site binds with the anions to be assayed. Injection of larger amounts of anions gives rise to a displacement of papaveraldinium which is no longer proportional to the amount injected. Moreover, the detection mechanism results from a dynamic equilibrium between anions and the detection agent forming ion pairs. This equilibrium is governed by the mass equilibrium law. Hence a competition for binding with papaveraldinium occurs between the different anions studied. The overload effect causes a decrease in the *DR* for other anions and thus a decrease in peak height. This could explain the observations concerning peak height reported by Barber and Carr[4]. Indeed, they noticed a decrease in retention time and an unproportional decrease in peak height when the amount injected was increased (amounts ranging from 5 to 50 nmol were injected). One could reduce the overload effect by increasing the concentration of papaveraldine and thus the *DR*. However, this is inconsistent with the optimization of the L.O.D. Indeed, a minimum

TABLE VII
OVERLOAD EFFECT: SOLUTIONS B' AND B

Overload effect[a]	*NaCl concentration (M) (B')*					*NaCl concentration (M) (B)*			
	$5 \cdot 10^{-5}$	$10 \cdot 10^{-5}$	$20 \cdot 10^{-5}$	$50 \cdot 10^{-5}$	$100 \cdot 10^{-5}$	$5 \cdot 10^{-4}$	$10 \cdot 10^{-4}$	$20 \cdot 10^{-4}$	$50 \cdot 10^{-4}$
X	0.035	0.065	0.134	0.312	0.672	0.0328	0.0468	0.105	0.256
Y	0.05	0.1	0.2	0.5	1	0.05	0.1	0.2	0.5
X/*Y*	0.698	0.646	0.671	0.624	0.672	0.656	0.468	0.526	0.512

[a] X = Cl^- peak area/NO_3^- peak area; Y = Cl^- concentration/NO_3^- concentration.

TABLE VIII
MECHANISM OF OVERLOAD EFFECT

Chloride concentration (M)	*DR (10^{-4} M)*		*DR (A) − DR (B) (10^{-4} M)*
	Sol. A	*Sol. B*	
$5 \cdot 10^{-4}$	11.22	7.59	3.63
$10 \cdot 10^{-4}$	9.97	8.01	1.96
$20 \cdot 10^{-4}$	9.62	7.89	1.72
$50 \cdot 10^{-4}$	8.39	7.16	1.23

L.O.D. can be obtained by decreasing the concentration of the detection agent as given by[10,11]

$$\text{L.O.D.} = \frac{C_m}{R \cdot DR}$$

where C_m is the IIR concentration in the mobile phase and R the dynamic reserve. The only way to obtain a minimum L.O.D. is therefore to increase the dynamic reserve.

Bromide blood assay

The treatment of epilepsy with bromides has recently been reintroduced for children for whom classical treatment has failed[13]. The therapeutic efficiency zone is narrow (20–25 mM)[8] and, further, it is necessary to determine the dosage for each epileptic syndrome. Hence the availability of a bromide blood assay with a millimolar sensitivity would be of interest. A small modification of the technique described above allows such an assay.

The retention time of bromide is 10 min and the limit of detection, coefficient of variation and selectivity are similar to those described above. The calibration graph for whole blood is linear from 5 to 50 mM. Fig. 4 shows a chromatogram corresponding to a 35-day treatment with a mixture containing sodium, potassium and ammonium bromides in equal parts. The peak corresponding to the normal Cl^- blood level appears at $t_R = 9$ min. Valproic acid, often used in the treatment, did not interfere with other anions because of its high pK_a with respect to the pH of the mobile phase.

Water analysis

Table I gives concentration limits for five anions in haemodialysis water (European Pharmacopoeia) with the corresponding L.O.D. using our method. This is sensitive enough to quantify concentrations near the limits for chlorides, nitrates, phosphates and sulphates. The existence of the overload effect implies that accurate quantification of an unknown sample containing many anions is difficult and requires a calibration using the standard addition method.

CONCLUSION

Some interesting applications are possible using this method, which permits the separation of seven anions in 20 min. The usable working range is $2 \cdot 10^{-5}$–$2 \cdot 10^{-4}$

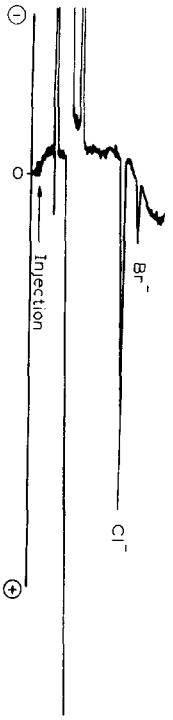

Fig. 4. Blood bromide assay. Bromide concentration, 10 m*M*.

M with an overloading anion concentration of 10^{-3} *M*. In water analysis, performing a limit test would simply consist of comparing the sample with a standard solution containing phosphate, chloride, nitrate and sulphate anions at the limiting concentration. The method is suitable for blood bromide assays because of the low anion concentration in the diluted blood sample. This method is already being used for pharmaceutical and clinical studies.

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