Journal of Pharmaceutical & Biomedical Analysis
Vol. 2, Nos 3/4, pp. 465–471, 1984
Printed in Great Britain

0731–7085/84 $3.00 + 0.00

Stability-indicating determination of adrenaline in injections containing procaine

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Abstract: Adrenaline was determined in injections containing procaine in a 1000-fold excess by reversed-phase high-performance liquid chromatography using UV detection at 205 nm and aqueous sulphuric acid (100 μmol/l) as eluent. The relative standard deviation was 2.1%, and the method was selective in the presence of adrenaline degradation products. Changes of the capacity factor with pH and ionic strength of the eluent were studied, and a simple model is suggested to explain the retention data.

Keywords: *Adrenaline; procaine; high-performance liquid chromatography; reversed-phase retention model.*

Ultraviolet spectrophotometric, colorimetric or fluorimetric methods [1] are generally not suitable for the assay of adrenaline (epinephrine) in the presence of its degradation products, or of other formulation components. High-performance liquid chromatography (HPLC) has been used for the separation of adrenaline from other catecholamines [2, 3] and from its degradation products [4–7]. There appears to be no method suitable for the selective determination of adrenaline in injections containing procaine in a 1000-fold excess, and for the simultaneous detection of the degradation products. The therapeutic efficacy of such preparations depends on the quantity of intact adrenaline, for an appreciable decrease of the local anaesthetic action is caused by its degradation. This paper describes a stability-indicating HPLC method for the selective determination of adrenaline in procaine injections. A simple mathematical model describes the reversed-phase HPLC retention behaviour of adrenaline in aqueous acid eluents.

Experimental

Chemicals and reagents

Adrenaline (Merck, FRG) and analytical grade chemicals and solvents (Reanal, Hungary) were used. Injections containing 5.0 μg/ml adrenaline and 5.0 mg/ml procaine were prepared in a hospital pharmacy or in the authors' laboratory. The water used as eluent was doubly distilled and stored in a glass container. To minimize degradation, the

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adrenaline stock solutions were prepared with a deoxygenated 50 mmol/l boric acid solution in an atmosphere of nitrogen. For the investigation of its degradation products, adrenaline was dissolved in 0.1 mol/l sodium hydroxide, and the solution allowed to stand in an open vessel for 30 min, when it was neutralized with hydrochloric acid.

Equipment

A Perkin–Elmer Model 601 liquid chromatograph fitted with a Perkin–Elmer ODS-Sil-X-1 column (300 × 2.6 mm i.d.), and a Perkin–Elmer variable wavelength detector were used. The full scale deflection of the recorder corresponded to approximately 0.033 absorbance unit/250 mm.

Chromatographic conditions

The column temperature was 35°C. The flow rate was 1 ml/min at an inlet pressure of *ca* 600 psi for aqueous eluent, and up to 1500 psi for a water–methanol (1:1 v/v) mixture. In early experiments various methanol–water mixtures (up to 50 vol % methanol), containing 0.5 mmol/l sulphuric acid, were used. The pH of the eluents was adjusted with sulphuric or acetic acid to the desired value (pH 2.8–6.2). The column was equilibrated with the eluent until the pH of the inlet and outlet solvent was the same. In certain cases the ionic strength (I) of the eluent was adjusted with sodium sulphate or perchlorate.

Results

Eluent composition

Various reversed-phase ion-pair systems have been proposed for the HPLC of adrenaline using water–methanol [5–7] or water–acetonitrile [7] mixtures, with sodium heptanesulphonate [5], hexanesulphonate [6] or dodecylsulphate [4, 7] as ion-pair reagents and acetic [5–7] or sulphuric [4] acids in the eluent. Sulphate and acetate have also been described [2] as ion-pairing reagents for the protonated adrenaline. No general retention model has been proposed to take into account the effects of pH, ionic strength, or the concentrations of organic modifiers.

Figure 1 shows the capacity factor dependence upon the methanol content of the eluent used in initial studies. The k' value decreases markedly for procaine and

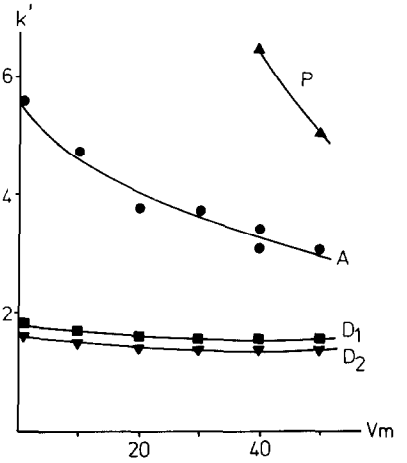

Figure 1
The effect of methanol (V_m-ml in 100 ml mixture) on capacity ratios (k') of procaine (P), adrenaline (A) and its two primary degradation products (D_1, D_2) in aqueous eluent containing 0.50 mmol/l sulphuric acid. Detection wavelength: 279 nm.

moderately for adrenaline with increasing methanol content. By contrast the capacity factor of the adrenaline degradation products remained almost unaffected. On the basis of these results, acidic aqueous eluents were then investigated. In order to develop the optimum chromatographic conditions, the effects of pH and ionic strength on the k' of adrenaline were studied. Figure 2 shows that when only acids were used for the pH adjustment ($I \sim 0$), the data for both sulphuric and acetic eluents followed the same sigmoid curve (Fig. 2A). Rather surprisingly, at a slightly higher ionic strength (Fig. 2B), lower k' values were obtained for both the acetic acid–sodium perchlorate eluent and the sulphuric acid–sodium sulphate eluent.

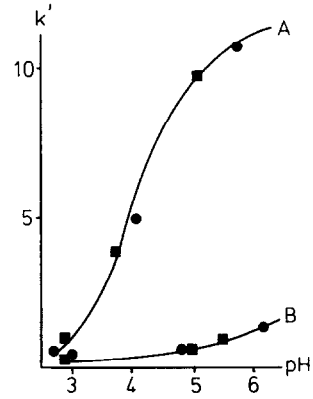

Figure 2
Capacity ratios of adrenaline using aqueous acid eluents of different pH and ionic strength. (A) Eluents, containing only sufficient acetic acid (●) or sulphuric acid (■) for pH adjustment ($I \sim 0$). (B) Eluents of $I = 0.03$ ionic strength; ●, acetic acid–sodium perchlorate; ■, sulphuric acid–sodium sulphate. Continuous line: calculated curves derived on the basis of the suggested retention model (see text).

For a reasonable analysis time and separation, the value for k' should be 1–5. Thus, an aqueous acid solution of pH 3–4 and $I \sim 0$ was used for the reversed-phase HPLC of adrenaline in procaine injections, the retention time for procaine being *ca* 20 min. At this pH, no degradation of column performance was observed.

In this early work, the wavelength selected for detection was 279 nm, where the molar absorptivity of adrenaline is 2600 l/mol cm [6]. However, this wavelength permits the assay of adrenaline only in the 5–15-μg range [6], which in the procaine–adrenaline injections is associated with as much as 5–15 mg procaine. Adrenaline has two more UV maxima in acid solutions at *ca* 224 and 205 nm, with two- and ten-fold greater molar absorptivities respectively than that at 279 nm. Preliminary experiments showed that the simple aqueous acid eluent was sufficiently transparent to permit the detection of adrenaline at 205–210 nm. Consequently, very dilute adrenaline solutions could be analysed. Procaine could also be detected at 205–210 nm, but did not interfere with the analysis of adrenaline as it remained bound to the column. To remove it the column was flushed with eluent for 40 min after three consecutive analyses.

Analytical Results

A 20-μl aliquot of an injection (equivalent to 100 ng adrenaline) with co-formulated procaine, boric acid, sodium chloride and sodium pyrosulphite, was injected into the chromatograph and eluted with aqueous eluent containing 100 μmol/l sulphuric acid (pH ~3.7). To assess the linearity of the analytical response 19 determinations were carried out in the range 40–450 ng adrenaline on different days. The correlation coefficient (r) was 0.9901. The following statistical parameters were calculated for the interassay variation: $h = bm + a$; $b = 0.253 \pm 0.031$; $a = 3.9 \pm 4.2$, where h is the peak height (in

mm), m is the mass of adrenaline in ng and a is the intercept. The errors given are the 95% confidence intervals. The regression was linear and regressed through the origin.

Because of the relatively high interassay variances, the calibration graph was constructed in the range 50–250 ng from 5–7 consecutive analyses every day. The observed intra-assay variances (confidence intervals at $p = 0.05$ were <0.006 for b and <0.1 for a, with $r > 0.999$) were used when the adrenaline content of the analysed samples was calculated from the data of 5–10 experiments. The RSD calculated from nine consecutive experiments with 100–200 ng adrenaline was 2.1%. To check the accuracy of the method, the recovery of adrenaline from an injection prepared in the laboratory was determined. The recovery was found to be 99.7 ± 1.6% ($p = 0.05$; $n = 9$) indicating no significant bias. Experiments were also carried out by ion-pair reversed-phase HPLC with an eluent system previously described for the investigation of catecholamines [8] and a home-made electrochemical detector [9]. The results did not differ significantly (Student's t-test, $p = 0.05$) from those measured by the present UV method.

Figure 3 demonstrates the suitability of the method as a stability-indicating assay of adrenaline. It should be noted that in the aged injections other degradation products (D_3 and D_4, Fig. 3) dominated, rather than the primary products of decomposition in sodium hydroxide solution (D_1 and D_2, Figs 1 and 2). Injections prepared without the use of nitrogen showed a rapid decrease in the adrenaline content (66–70% of the original content was lost in 24 h, and 47–51% in 72 h), with increasing peaks for the degradation products. Three-month-old injections prepared in a nitrogen atmosphere contained only 44–48% of the original adrenaline. These results indicate the instability of the adrenaline formulation and the importance of a rapid, selective assay for adrenaline in local anaesthetic injections.

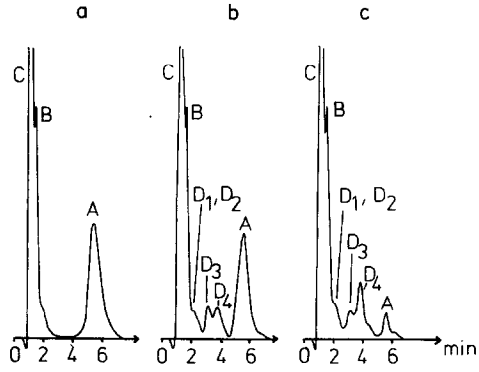

Figure 3
Chromatogram of a procaine–adrenaline injection (150 ng adrenaline chromatographed with 100 μmol/l sulphuric acid eluent). (a) Freshly prepared, (b) after 1 month storage, (c) after 5 months storage. (A) adrenaline; D_1, D_2, D_3, D_4 unidentified adrenaline degradation products; (B) sodium pyrosulphite; (C) contaminants from the inorganic components of the injection. Procaine remained on the column. Detection wavelength: 205 nm.

Discussion

In a reversed-phase chromatographic system the degree of protonation of a compound can be taken as equal to that calculated with the macroscopic dissociation constant [2], but the retention of the compound is mainly dependent on its hydrophobic nature [2, 5]. Using $pK = 8.8$ for procaine [10], and $pK_1 = 8.66$, $pK_2 = 9.95$ for adrenaline [11], both compounds should be present as cations (Fig. 4) in solution at pH <6. By virtue of its alkyl groups, the procaine cation would be expected to be the more hydrophobic compound. No pH-dependence of k' would be expected with eluents of pH <6, in contrast to the experimental data presented for adrenaline (Fig. 2). No pH-dependence

of k' was observed by Asmus and Freed [2] for certain catecholamines in acid–salt mixtures in the pH range 2–5. However, the ionic strength of their eluents was higher than that used in the present work. Figure 2 shows that the pH-dependence of k' in solutions of very low ionic strengths ($I \sim 0$; Fig. 2A) was suppressed by the salt effect (Fig. 2B). Similar results were observed with sulphuric acid–sodium sulphate and acetic acid–sodium perchlorate systems. This does not support the ion-pair-forming characteristics of sulphate and acetate ions previously suggested [2]. An alternative explanation is required.

The reversed-phase HPLC retention of small hydrophobic solutes is generally described as a liquid–liquid partition in the bonded alkyl phase, but that of solute molecules with polar substituents is considered to involve adsorption on the uncoated parts of the stationary phase [12]. However, considering the shape of the k' versus pH curves (Fig. 2), a pure partition model can also be proposed to explain the retention behaviour of adrenaline with aqueous acid eluents in the absence of organic modifiers. This model is based on the absorption theory of drugs and utilizes the microscopic dissociation constants of adrenaline.

According to the absorption theory proposed by Wagner [13], the rate of phase transfer is controlled mainly by the distribution of the essentially uncharged species (HA^0), illustrated in Fig. 4. The higher the distribution coefficient, the greater the shift observed for its mole fraction versus pH curve towards the acidic region [13, 14]. Assuming that the capacity factor k' is proportional to the mole fraction of adrenaline in the stationary phase, this model is also suitable for the description of the k' versus pH experimental curves. Using a Hewlett–Packard HP41C computer and literature values of the microscopic dissociation constants k_1, k_2, k_{12}, k_{21} (Fig. 4) of adrenaline [11], mole fraction versus pH curves with different distribution coefficient values were calculated and their characteristics compared with the experimental data. The best fit was observed when the distribution of the H_2A^+ cation was also taken into account.

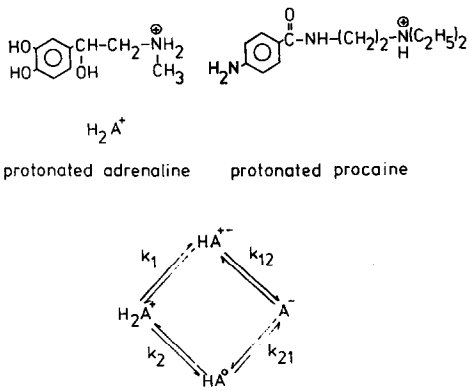

Figure 4
Structural formulae of protonated adrenaline and procaine, and the microscopic deprotonation process of the adrenaline cation.

If k' is directly proportional to the sum of the mole fractions of adrenaline in the stationary phase:

$$k' = C'(x^{st}_{HA^0} + x^{st}_{H_2A^+}) = C' \frac{K^D_{HA^0}[HA^0] + K^D_{H_2A^+}[H_2A^+]}{c_A}, \quad (1)$$

$$K^D_{HA^0} = \frac{[HA^0]_{st}}{[HA^0]}, \; K^D_{H_2A^+} = \frac{[H_2A^+]_{st}}{[H_2A]}, \quad (2)$$

where C' is an arbitrary constant, $x^{st}_{HA^0}$ and $x^{st}_{H_2A^+}$ are the mole fractions in the stationary phase, $K^D_{HA^0}$ and $K^D_{H_2A^+}$ the distribution coefficients of the HA^0 and H_2A^+ species, respectively; $[H_2A]$ $[H_2A^+]$ and $[HA^0]$ denote concentrations in the aqueous eluent; $[HA^0]_{st}$ and $[H_2A^+]_{st}$ are concentrations in the stationary phase; and c_A denotes the total molar concentration of adrenaline:

$$c_A = [H_2A^+] + [HA^{+-}] + [HA^0] + [A^-] + [H_2A^+]_{st} + [HA^0]_{st}. \quad (3)$$

Using (1)–(3), the individual concentrations, the microscopic dissociation constants and $[H_2A^+]$, it can be shown that

$$k' = C' \frac{K^D_{HA^0} k_2 [H^+] + K^D_{H_2A^+} [H^+]^2}{k_1 k_2 + [H^+][k_1 + k_2(1 + K^D_{HA^0})] + [H^+]^2 (1 + K^D_{H_2A^+})},$$

Data used for the 'best' calculated curves, shown in Fig. 2, are: A curve: $\log K^D_{HA^0} = 5.5$, $\log K^D_{H_2A^+} = -1.7$, $C' = 10.6$; B curve: $\log K^D_{HA^0} = 2.5$, $\log K^D_{H_2A^+} = -1.75$, $C' = 10.6$.

The value of $K^D_{HA^0}$ for the $I \sim 0$ system, and its ionic strength dependence, are higher than expected for a very polar solute. The reversed-phase HPLC retention, however, can hardly be described as a thermodynamic phase equilibrium established after extraction. It may be similar to the process of emulsification existing during extraction, when phases are partially mixed by shaking. As a consequence of the flow of the polar phase, eluent components are not only adsorbed but may also be 'emulsified' in the non-polar stationary phase. Yonker *et al.* [15] recently proposed that water molecules could be bound on the surface of C_{18} stationary phase not only to the residual silanols, but also due to trapping by a 'tent' of the alkyl chains. The water molecules, being hydrogen-bonded donors, would carry other polar molecules with them into the stationary phase [15]. This picture is very similar to the explanation based on an 'emulsifying process' discussed above. The 'other' phase of the distribution for polar solutes may be not only the phase containing the hydrophobic carbon chains, but also the water 'emulsified' in the 'stationary phase' (Fig. 5). The exchange of water molecules is facilitated by the constant flow of eluent, permitting the distribution of other organic molecules. The

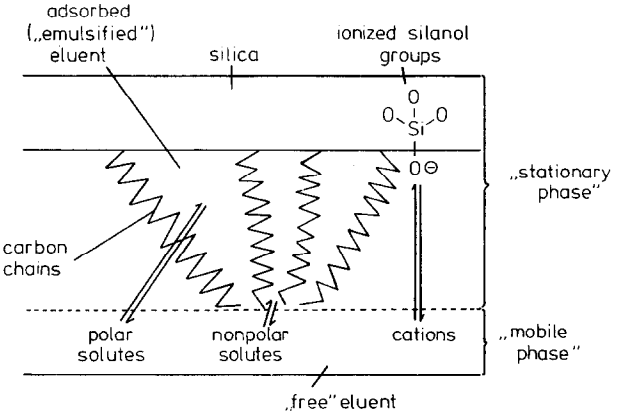

Figure 5
Model proposed for the reversed-phase HPLC retention of adrenaline.

adrenaline, bound to water molecules trapped in a C_{18} alkyl chain tent, can also interact with the chains by means of its hydrophobic moiety.

This process, similar to the formation of an emulsion, is more dependent on ionic strength than a 'real' liquid–liquid partition. This fact may explain the observed partition coefficient value and its marked decrease with increasing ionic strength.

In this model the (very restricted) distribution of the protonated H_2A^+ adrenaline can be explained by the ion–ion interaction of the cation with the residual silanol groups, deprotonated in solution at pH >2. The lower ionic strength dependence of the partition coefficient for H_2A^+ may support this assumption.

It can be seen that all the experimental data could be explained by the simple retention model discussed above. Thus, the experimentally optimized eluent composition, pH and ionic strength have also been supported on a physicochemical basis. These results suggest that simple aqueous acid eluents can conveniently be used without organic modifiers in reversed-phase HPLC. The application of such eluents permits the use of protonation and other formation constants, generally available only for aqueous solutions, for the study of the equilibria occurring during elution.

Acknowledgements: Thanks are due to Mr László Szücs for the determinations using the electrochemical detector, and to Mrs Rita Hartmann for her valuable technical assistance.

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[*First received for review June 1982; revised manuscript received 2 August 1982*]