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RETENTION BEHAVIOUR OF A BONDED REVERSED PHASE IN A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF SERUM THEOPHYLLINE

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SUMMARY

A simple, rapid assay for serum theophylline is described. Optimisation of the high-performance liquid chromatographic system has allowed the analysis of minimum sample volume (50 μl) in the shortest time (8 min) with good between-batch precision (C.V. = 8.3%) and accuracy (recovery *ca.* 100%). The behaviour of theophylline and other xanthine derivatives, theobromine, caffeine and 8-chlorotheophylline, on a bonded octadecyl reversed-phase system suggests that the C_{18} phase effects solute separation by mixed retention mechanisms rather than by pure reversed-phase chromatography. This behaviour is attributed to the participation of underivatised silanol groups in the chromatographic process, an observation corroborated by independent investigation. The mechanism of the silanol participation is difficult to rationalise; however, a normal-phase liquid partition system complies with the experimental data.

INTRODUCTION

The routine determination of serum theophylline levels is now operational in many clinical laboratories. Recently, chromatographic methods of high sensitivity and selectivity have been reported[1–10]. Gas chromatographic (GC) methods require tedious extraction procedures and usually sample derivatization prior to analysis[7–9]. High-performance liquid chromatography (HPLC) requires minimal sample preparation making it an attractive approach for routine use[1–6]. However, interactions between solutes, stationary and moving phases make the optimization of liquid chromatographic separations more difficult than those in GC which essentially lack moving phase interactions. For this reason, thorough investigation of any method is warranted prior to arriving at an optimal separation.

We report an HPLC method for which only 50 μl of plasma is required with 8-chlorotheophylline as the internal standard. The behaviour of the chromatographic system has been investigated in an attempt to arrive at optimal conditions. Evidence is presented which suggests that an octadecylsilane phase bonded to silica particles acts via mixed retention mechanisms probably due to the action of free silanol groups, rather than by pure reversed-phase partition.

EXPERIMENTAL

Apparatus

The ALC Model 202 liquid chromatograph consisted of a Model 6000A 41.4-MPa (6000 p.s.i.) pump, a U6K high-pressure injector system and a fixed-wavelength (280 nm) Model 440 absorbance detector equipped with a 12-μl flow cell (all from Waters Assoc., Milford, Mass., U.S.A.).

Chromatographic conditions

A 30 cm $\times$ 4 mm I.D. stainless-steel column was packed with a stable reversed-phase stationary phase consisting of porous silica beads (mean diameter 10 μm) coated with a chemically bonded monolayer of octadecylsilane (μBondapack C_{18}; Waters Assoc.). The mobile phase was 1% propionic acid (adjusted to pH 5.0 with 2 *N* NaOH)–methanol (80:20, v/v). The operating temperature was ambient and the flow-rate 1.7 ml/min, with an operating pressure of 17.25 MPa (2500 p.s.i.). The column effluent was monitored continuously at 280 nm, with a full-scale deflection of 0.02 *A*. A short methanol wash (20 min at 1 ml/min) at the end of each analytical day was included to remove strongly retained solutes. Each month, the system was routinely flushed with methanol, then acetonitrile, then chloroform and finally hexane and back to methanol by a reverse series.

Reagents

All chemicals were reagent grade. Methanol was Chrom AR grade (Mallinckrodt, St. Louis, Mo., U.S.A.). Theophylline and theobromine were donated by Parke-Davis (Brockville, Canada). Caffeine and 8-chlorotheophylline were purchased from Aldrich (Milwaukee, Wisc., U.S.A.). Solvents are routinely filtered through 0.45-μm filters (Millipore, Bedford, Mass., U.S.A.) prior to use in the liquid chromatograph.

Standards

Theophylline (20 mg) was dissolved in distilled water (100 ml). This solution was used to prepare plasma standards containing 20, 10 and 5 μg/ml (110, 55 and 27.5 μmoles/l, respectively). Aliquots (*ca.* 200 μl) were stored at $-20°$ prior to use in an analytical run. For the internal standard, 8-chlorotheophylline (10 mg) was dissolved in chloroform (100 ml). 1 ml of this solution was made up to 1 l with chloroform and this solution served as the extraction solvent.

Extraction procedure

Serum or plasma (50 μl) was added to a 40-ml glass tube fitted with a PTFE lined screw cap. Chloroform (10 ml) containing the 8-chlorotheophylline internal standard was added, followed by sodium chloride (*ca.* 1 g). Extraction during 10 min (Buchler Omnishaker) was followed by centrifugation at 2000 rpm for 2 min (350 *g*). The small aqueous phase was removed by aspiration, the chloroform decanted into a conical tube (15 ml) and taken to dryness by warming under a stream of dry nitrogen. The residue was dissolved in methanol (*ca.* 40 μl) and 25 μl was injected into the liquid chromatograph. This procedure was followed for patient and standard samples and the peak height ratio of theophylline and 8-chlorotheophylline was determined.

RESULTS AND DISCUSSION

Theophylline assay

A typical chromatogram of a patient sample is shown in Fig. 1. The analysis of the plasma standards showed that the relationship between plasma concentration of theophylline and the peak height ratios of theophylline and 8-chlorotheophylline is linear between 5 and 20 μg/ml. Table I reports precision and recovery data. These results confirm the reliability and accuracy of the method.

TABLE I

PRECISION AND RECOVERY DATA

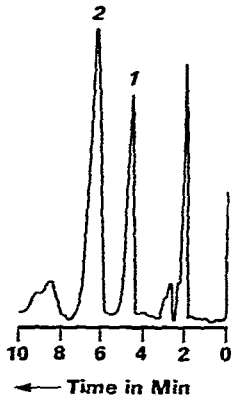
S.D. = standard deviation; C.V. coefficient of variation.

Precision (pool sample)			*Final plasma conc. (μg/ml)*	*Conc. found by assay (mean N = 3)*	*Recovery* (%)*
Mean (N = 30)	*S.D.*	*C.V.*			
12.0	1.0	8.3	2	2.1	105
			5	5.0	100
			10	9.5	95
			20	19.6	98

* All recoveries lie within the between-bath coefficient of variation (8.3%).

Other features also make this procedure attractive for routine use: the 50-μl sample requirement enables analysis of samples from neonatal patients, enhancing the management of neonatal apnea. The sensitivity and small sample requirement facilitate pharmacokinetic investigations. The sample extraction procedure and short elution time (*ca.* 8 min) allow rapid analyses. The mobile phase is predominantly water-based, easily prepared, cheap and presents little laboratory hazard. There is no tedious column regeneration procedure and the routine precautions outlined above obviate the need for replaceable pre-columns.

Any chromatographic process, preceded by a simple extraction of a biological fluid is open to interferences from endogenous compounds and from other drugs which may be present and elute with the material of interest. Theophylline is a weak base ($pK_a = 8.8$)[11] and can be extracted into chloroform with good recovery at neutral pH. Under these conditions, however, strongly basic and acidic material will remain in the aqueous phase and will not interfere with subsequent chromatography. Barbiturates, which are often used with theophylline, are extracted at neutral pH; their detector response, however, at 280 nm and pH 5.0 is negligible. Other xanthine derivatives used as therapeutics, notably theobromine (1,7-dimethylxanthine) and caffeine (1,3,7-trimethylxanthine) may interfere with the analysis. A chromatogram of a sample containing theophylline, 8-chlorotheophylline, theobromine and caffeine is presented in Fig. 2, showing baseline separation of the four solutes with a total elution time of less than 10 min. An exhaustive investigation of other materials which may interfere has not been undertaken.



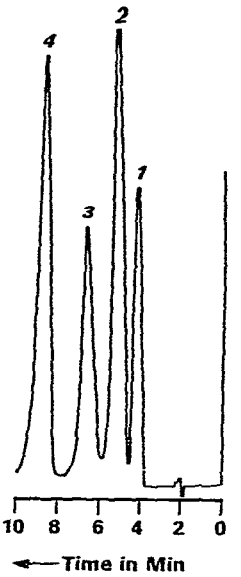


Fig. 1. Chromatogram of patient sample. Solvent, 1% propionic acid–methanol (80:20) adjusted to pH 5.0 as described in the text; flow-rate, 1.7 ml/min. 1 = Theophylline, 2 = 8-chlorotheophylline.

Fig. 2. Chromatogram of theobromine (1), theophylline (2), 8-chlorotheophylline (3) and caffeine (4). Solvent, 1% propionic acid–methanol (80:20) adjusted to pH 5.0 as described in the text; flow-rate, 1.7 ml/min.

Behaviour of the C_{18} reverse phase

Fig. 3 shows a chromatogram of 8-chlorotheophylline, theobromine, theophylline and caffeine produced by the mobile phase water–methanol (86:14), with capacity factors k' of 0, 2.1, 3.1 and 7.4, respectively (Table II; pH = 6.0). The relative positions of caffeine, a trimethylxanthine, and the dimethyl stereoisomers might be rationalised using a reversed-phase partition argument. However, the zero retention

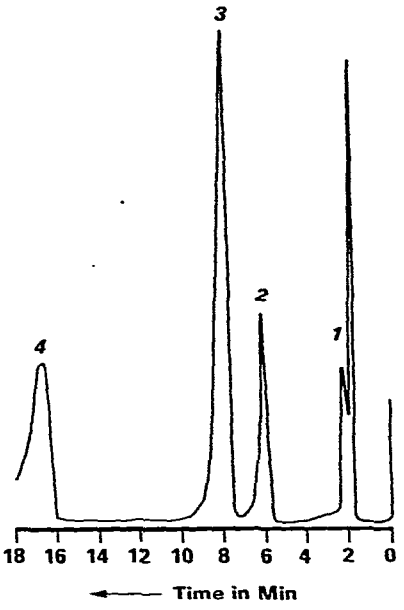
TABLE II

THE VARIATION OF k' AND α VALUES WITH SOLVENT pH

$\alpha_1 = k'_2/k'_1$; $\alpha_2 = k'_3/k'_2$; $\alpha_3 = k'_4/k'_3$.

pH	*Theobromine*		*Theophylline*		*8-Chlorotheophylline*		*Caffeine*
	k'_1	α_1	k'_2	α_2	k'_3	α_3	k'_4
3.0	1.7	1.5	2.5	2.1	5.3	1.2	6.2
4.0	1.7	1.5	2.5	2.0	5.0	1.2	5.9
4.5	1.7	1.5	2.5	1.9	4.8	1.2	5.8
4.75	1.7	1.5	2.5	1.8	4.6	1.3	5.8
5.0	1.8	1.5	2.7	1.5	4.1	1.5	6.0
5.5	2.0	1.4	2.8	1.1	3.0	2.1	6.4
6.0	2.1	1.5	3.1	—	0	—	7.4
5.0*	1.0	1.5	1.5	1.4	2.2	1.5	3.3

* 20% Methanol.



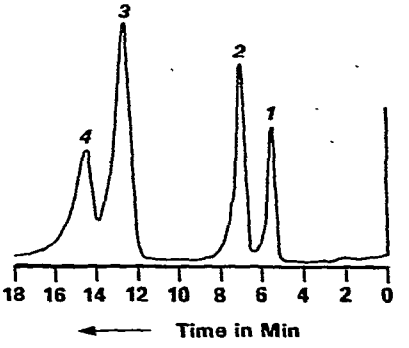


Fig. 3. Chromatogram of 8-chlorotheophylline (1), theobromine (2), theophylline (3) and caffeine (4). Solvent, water–methanol (86:14; pH, 6); flow-rate, 1.7 ml/min.

Fig. 4. Chromatogram of theobromine (1), theophylline (2), 8-chlorotheophylline (3) and caffeine (4). Solvent, 1% propionic acid–methanol (86:14); pH, 3.

of 8-chlorotheophylline is not supported by this argument. This chloro derivative should be less basic than theophylline due to inductive and field effects exerted by the chlorine at C_8 on the lone pair of N_7, and whereas the pK_a values of caffeine (pK_a = 14)[11], theobromine (9.9)[11] and theophylline (8.8)[11] suggest that they will hold a positive charge in the water–methanol (86:14) system, it is likely that the chloro derivative will be present as a neutral species. Greater retention of charged moieties over that observed for neutral species is, however, uncharacteristic of a pure reversed-phase partition system and suggests that the solutes may be interacting with residual silanol groups in the stationary phase.

This argument predicts an increase in k' for 8-chlorotheophylline if it is converted to a cation species, and Fig. 4 shows the chromatogram in 1% propionic acid–methanol (86:14). The value of k' for 8-chlorotheophylline is now 5.3, whereas, as expected, k' values of the other three solutes are virtually unchanged (Table II; pH = 3.0). This effect suggests that an intermediate pH in this solvent would produce relative k' values such that the resolution of the four solutes is optimised and Table II shows k' values and α values of adjacent bands over a range of pH. Theophylline/theobromine, α_1, remains almost unchanged over the range whereas 8-chlorotheophylline/theophylline, α_2, and caffeine/8-chlorotheophylline, α_3, range from 2.2 to 1.1 and 1.2 to 2.1, respectively. Clearly the optimum position lies at the point $\alpha_2 = \alpha_3$, and this occurs close to pH 5.0 ($\alpha_2 = 1.52$, $\alpha_3 = 1.46$). Finally, by increasing the methanol concentration the respective k' values are reduced without affecting α

values (Table II) making the pH 5.0 1% propionic acid–methanol (80:20) the system of choice for biological analyses.

The effect of residual silanol groups has been documented for GC systems[12] and recently it has been shown that mixed retention mechanisms involving bonded alkyl reversed-phases and unchanged silica occurs in liquid chromatography[13]. Furthermore, it was shown that for solutes with small or non-existent alkyl portions (*e.g.*, the xanthine derivatives), the bonded alkyl phase serves mainly to deactivate the underlying silica surface[13], suggesting that residual silanol groups play an important role in the retention of such species. The mechanism of the silanol effect is difficult to rationalise, and it is evident from recent work that caution must be taken when discussing mechanisms on bonded reversed-phases without having first defined the quality of the stationary phase[14]. Nevertheless, simple qualitative arguments can be attempted to explain the mixed retention mechanisms evident in this work.

First, since the mobile phase is highly polar it is likely that adsorption effects can be discounted, moreover charged species would probably be strongly adsorbed leading to excessive retention volumes and tailing peaks. A cation exchange effect:

$$\text{–Si–O–H} + X^+ \rightleftharpoons \text{–Si–O–X} + H^+$$

could be rationalised. However, a reported pK_a value of 10.5 ± 1 (ref. 15) for silicic acid does not support this idea. Moreover, using an isoelectric point for silica in the pH range of 4–5, it has been reported that silica is negatively charged at higher pH, whereas, at lower pH it is neutral or even cationic[16]. Such behaviour suggests that silica may mimic the weakly acidic cation exchangers and thereby, show optimum exchange capacity at pH values >7 with minimal or zero capacity at pH ≤3. Since good retention of the xanthines occurs at pH 3.0, a cation exchange effect seems to be precluded. A reversed-phase ion-pair effect without a silanol contribution might be considered, in which the positively charged solutes pair with the propionate ion. However, since k' values for theophylline, theobromine and caffeine do not depend on the presence of propionic acid (Table II; pH 6.0) and since the same k' *vs.* pH titration for 8-chlorotheophylline can be shown when the pH is adjusted with a mineral acid (HCl) and simple organic acids, (acetic acid and formic acid) it is unlikely that the ion-pair mechanism operates.

The concept of partition retention mechanisms on silica is not new[17]. It has been shown that silica exposed to highly polar solvent mixtures will form a liquid stationary phase with the intermost layers rich in water and a gradient increase in organic content towards the outer layers[15]. Clearly the water dominated solvents used in this work may have such an effect with residual silanol groups, leading to a stationary liquid phase with increasing methanol concentration in the outer layers. The xanthine derivatives will be separated by a partition process between this polar stationary phase and now somewhat less polar moving phase. The behaviour of 8-chlorotheophylline with solvent pH is explained since in this normal phase system the k' value for a charged species should, as observed, exceed the k' of the neutral molecule.

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