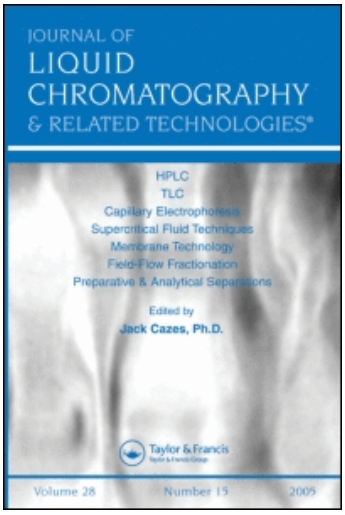

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SIMULTANEOUS MEASUREMENT OF ADENOSINE 5'-TRIPHOSPHATE AND ITS DEGRADATION PRODUCTS BY REVERSE-PHASE ION-PAIR CHROMATOGRAPHY

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ABSTRACT

A method is presented for the simultaneous measurement of adenosine 5'-triphosphate and its uv-absorbing degradation products by reversed-phase liquid chromatography using tetrabutylammonium phosphate as an ion-pair agent. The method is suitable for the measurement of these compounds in isolated smooth muscle.

INTRODUCTION

Adenosine and the adenine nucleotides have diverse and important roles in medicine and biology. This has led to a corresponding diversity of HPLC analyses tailored to the particular needs and interests of the investigator. These are reviewed in detail by Zakaria and Brown (1). The most frequent-

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ly used method for the analysis of nucleosides and bases has been separation on an ODS column with phosphate buffer/methanol eluents, while the more polar nucleotides have been separated by ion-exchange methods. Control of the subtleties of the separation has been by buffer composition and pH, the use of gradient separations and different columns. More recent papers report the use of ion-pair reagents to replace ion-exchange methods and there are a few reports (2-5) of simultaneous analyses of purine nucleotides, nucleosides and bases. Most of these involve isocratic separations of a few selected compounds.

However, there has been no method reported which allows simultaneous determination of ATP and all its uv-absorbing degradation products (ADP, AMP, AR, HR, H, X, and UA) in a reasonably short time. We present such a method, the application in smooth muscle preparations, and some of the problems associated with the use of the method.

METHOD

All separations were done on a Microbondapak C-18 column (300x5mm, Waters Associates, Milford USA,) protected by a guard column filled with pellicular ODS packing (Bondapak C-18 Corasil, Waters Associates). This had had previous use with phosphate buffer (0.067M, pH 6.5) and short exposure to ammonium phosphate (0.067M, pH 6.3) and dibutylammonium phosphate

(0.015M pH 6.20), all run in various proportions with methanol. The HPLC system consisted of two M6000A pumps controlled by a M660 gradient programmer, a U6K injector and a M440 dual-channel (254nm and 280nm) uv detector (all from Waters Associates) and an Omniscribe chart recorder (Houston Instruments). Aqueous solvents were made with water freshly collected from a water purification system (Milli-Q, Millipore Ltd, Bedford, USA). HPLC grade methanol was obtained from Waters Associates. All solvents were vacuum filtered through an appropriate filter (0.45micron pore size) immediately before use.

Standard compounds were obtained from Sigma Chemical Company (St Louis, USA). Standard solutions were made to approximately the required concentration and the actual concentration checked by double-beam spectrophotometry. Other chemicals were of at least analytical reagent grade unless stated. Tetrabutylammonium phosphate was obtained from Waters Associates (Pic-A, TM) and tetrabutylammonium hydroxide laboratory reagent grade from BDH (Poole, UK).

Sample Preparation

Frozen tissue samples were placed in a homogeniser tube pre-cooled in a dry-ice bath and 0.5ml 10% ice-cold TCA added. The tissue was homogenised (Ultra-turrax) Model TP 18/10, Janke and Kunkel, Kikawerk) and centrifuged to remove precipitated

protein. The supernatant was extracted with water-saturated diethylether until the pH was in the range 4-5. Recovery experiments were done by addition of a standard mix and analysis of the spiked and unspiked samples. The difference was compared to the known added amount of purine.

Abbreviations

HPLC, high performance liquid chromatography; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; AMP, adenosine 5-monophosphate; AR, adenosine; HR, inosine; H, hypoxanthine; X, xanthine; UA, uric acid; TBA, tetrabutylammonium phosphate; TCA, tricholoracetic acid.

RESULTS

Initial development work for this assay was done using Pic-A made up as suggested i.e. at 5mM. It was necessary to use a methanol gradient to elute all the compounds of interest in a reasonable time, and a linear gradient from 0-36% methanol in 18 min at a flow-rate of 1.5ml/min was used. The effect of pH on the capacity ratio k' is shown in Fig 1. This shows the best separation was obtained at pH 6.10 but careful pH control was necessary to ensure reproducible separations. The concentration of Pic-A was varied at pH 6.10 and the effects on k' are shown in Fig 2. This shows that the maximum effect is obtained at 5mM with no extra benefit from the higher con-

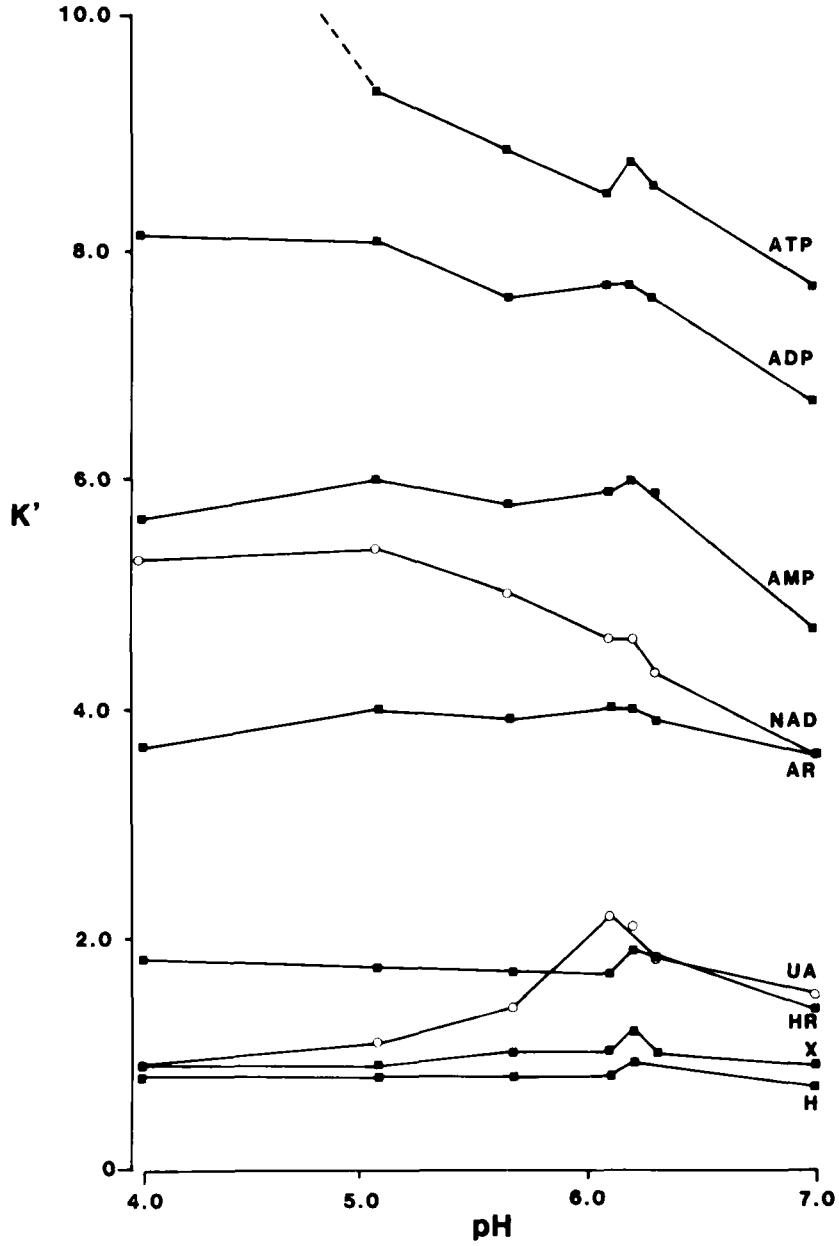

Figure 1. Variation in capacity ratio k' of the purines ATP, ADP, AMP, AR, HR, H, X, UA and NAD with altered mobilephase pH at 5mM TBA (Pic-A). Separation was obtained with a linear gradient to 36% methanol/5mM TBA in 18min at 1.5ml/min.

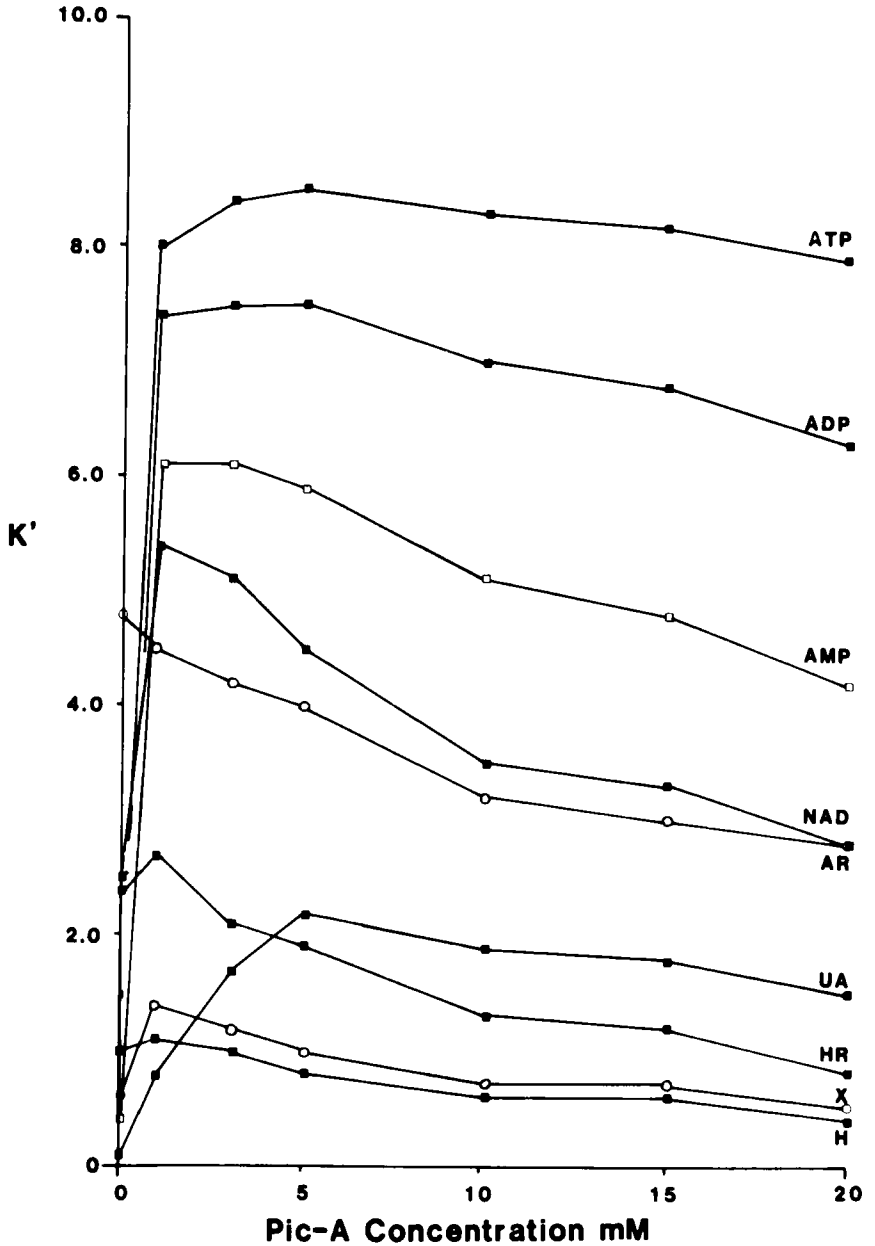

Figure 2. Variation in k' with altered TBA concentration. Separations were done at pH 6.10. Other conditions were as for Figure 1.

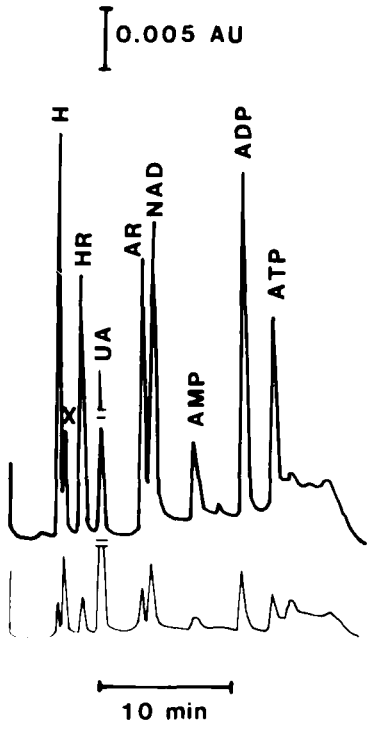

Figure 3. Chromatogram obtained with the selected operating conditions (5mM TBA, pH 6.10, linear gradient from 0-36% methanol in 18min at 1.5ml/min). The upper thicker line shows the absorbance at 254nm and the lower, thinner line shows the absorbance at 280nm. Injection was of approximately 1nmol of each purine.

centrations. The separation obtained finally is illustrated in Fig 3.

As peak height was linear with injected amount of purine, quantitation was made by peak height comparison with external standard solutions. These were run at least once each day. The system was used to separate and quantitate purines from

TCA extracts of guinea-pig ileum and rat vas deferens. Purines were identified by retention time, ratio of absorbance at 280nm to absorbance at 254nm and by enzyme peak shift in cases of doubt of peak purity.

The absorbance ratios are expressed as the mean ± the standard error of the mean of 6 observations. The coefficients of variation (C.V.) were obtained by extracting 6 pieces of tissue from the same animal and making a single injection of each extract. The recoveries were determined as described in the text.

The within-run coefficients of variation are shown in Table I together with the recovery data and the absorbance ratios of each purine. This shows that the recoveries are generally

TABLE I

Purines	Absorbance Ratios	Rat Vas deferens		Guinea-pig ileum	
	280:254nm	C.V.%	recovery%	C.V.%	recovery%
ATP	0.178±0.004	15	84	19	55
ADP	0.180±0.002	12	90	13	59
AMP	0.178±0.002	11	103	22	73
AR	0.180±0.002	11	93	6	97
HR	0.156±0.003	5	89	13	103
H	0.075±0.003	10	98	12	99
X	0.704±0.006	7	95	9	100
UA	2.629±0.031	8	86	13	107
NAD	0.231±0.002	11	90	17	100

good and the method reproducible for the samples under consideration. Chromatograms obtained from samples of guinea-pig ileum and rat vas deferens are shown in Fig 4A and 4B and the corresponding quantitation in Table II.

Purine content in pmol/mg wet weight of rat vas deferens and guinea-pig ileum extracted as in the methods. The < symbol indicates that the 280:254 absorbance ratio of H was different from that shown in Table I and therefore quantitation from the peak height produced a result greater than the amount of H actually present.

The analyses were proceeding well but it was felt that purchasing TBA made up in vials containing sufficient reagent to dilute to make one litre of solvent might be an unnecessary extravagance. We therefore made an equivalent solution from

TABLE II

Purine pmol/mg	Rat vas deferens	Guinea-pig ileum
ATP	47	96
ADP	84	463
AMP	82	343
AR	16	20
HR	1107	216
H	549	<181
X	305	191
UA	124	832
NAD	108	154

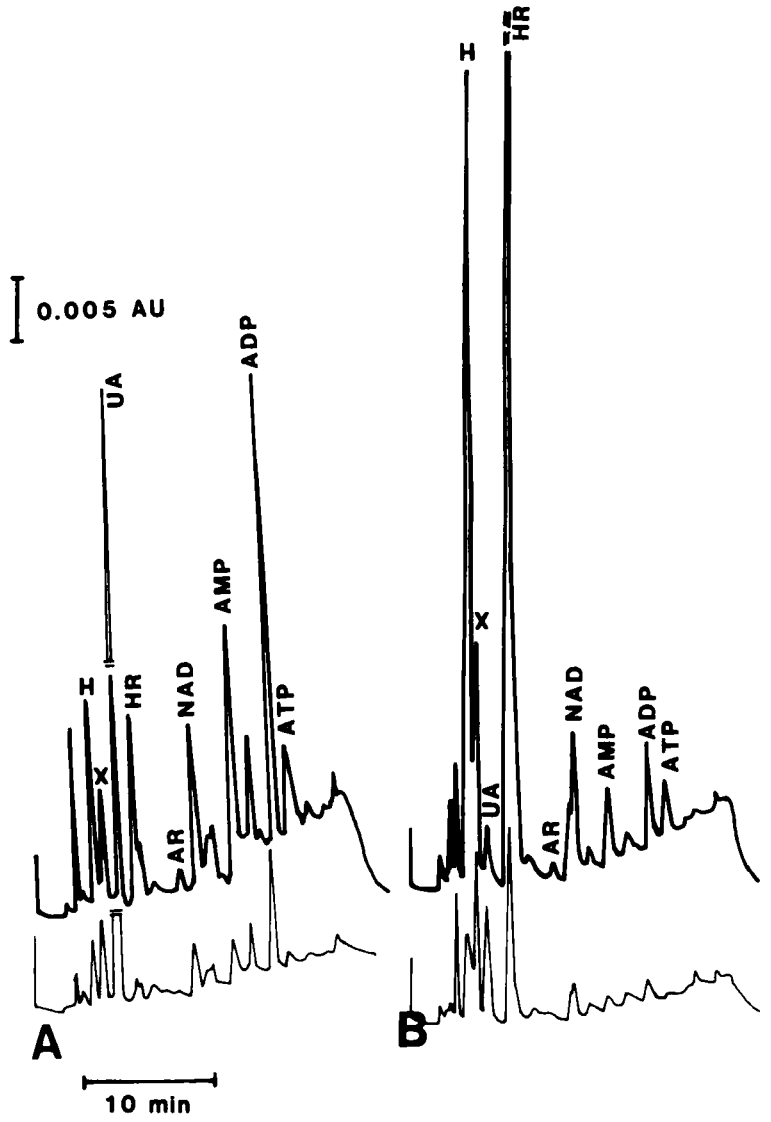

Figure 4. Chromatograms of smooth muscle extracts. Conditions are as given for Figure 3. A shows the chromatogram resulting from the injection of 0.015ml extracted guinea-pig ileum and B that resulting from the injection of 0.030ml of extracted rat vas deferens. The quantitations resulting from these chromatograms are presented in Table II.

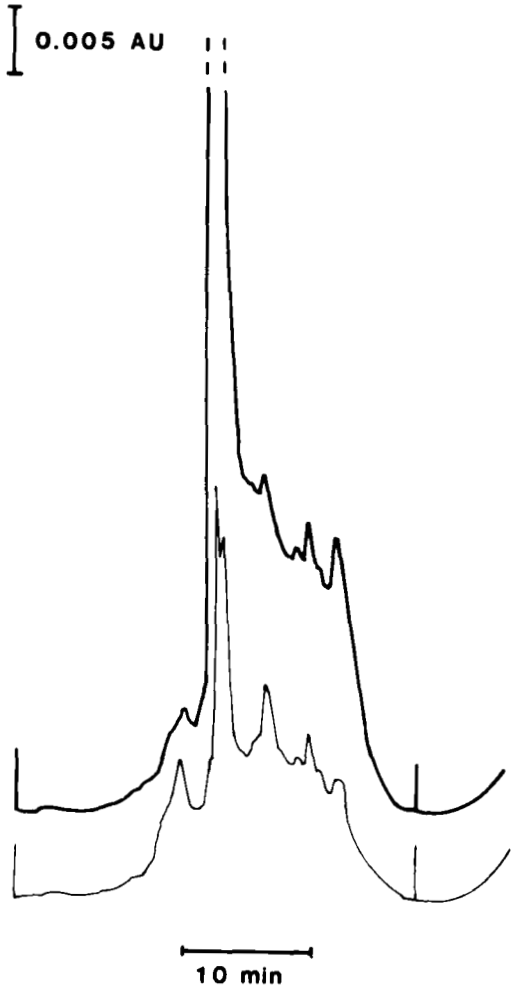

Figure 5. Baseline chromatogram obtained when the aqueous component of the mobile phase was TBA made from tetrabutylammonium hydroxide and phosphoric acid purified by passing through a Microbondapak column. Concentrations and other conditions were as given for Figure 3. The baseline returned to that seen in Figure 3 when the use of Pic-A was resumed.

tetrabutylammonium hydroxide and phosphoric acid. This produced separations as good as those obtained using the Pic-A. However, after a few runs the baseline began to rise, and the rises increased in magnitude with subsequent runs until it was no longer useable. The column was washed nightly in 40% methanol/water and each day the baselines showed the same rises. This was attributed to uv-absorbing impurities in the solvent absorbing onto the stationary phase at low methanol concentrations and subsequently being eluted as the methanol concentration increased. An attempt was made to purify the solvent by passing it through a Microbondapak column and guard-column combination at a flow-rate of 0.5ml/min. Following purification in this way the baseline improved considerably but was never as good as that obtained using Pic-A. Fig 5 shows one of the better chromatograms obtained using the "purified" TBA.

Dibutylammonium phosphate prepared as for tetrabutylammonium phosphate also showed steep baselines. In neither case did changes of chromatographic conditions or repeated washings with methanol/water/dimethylsulphoxide combinations make other than temporary improvements.

DISCUSSION

Ion-pair chromatography offers considerable advantages over ion-exchange chromatography of purine nucleotides, particularly since the high concentrations of salts (usually a

combination of phosphate and chloride) used to elute the higher nucleotides can be damaging to the HPLC hardware unless extreme care is taken in their use. Furthermore nucleosides and bases elute frontally on ion-exchange systems. Consequently to measure nucleotides, nucleosides and bases requires two separate chromatographic runs, whereas they are separated with the reversed-phase ion-pair systems.

Caronia et al (8) have studied the mechanism of separation of deoxyribonucleotides and deoxyribonucleosides using tetrabutylammonium hetaerons. They showed that as the concentration of hetaeron on the stationary phase increased, the capacity ratio of nucleotides increased and that of nucleosides decreased. This is also seen at the low concentration end of Fig 2. In addition the purine bases H and X behave like the nucleosides and the more ionic UA is retained longer on the column at higher hetaeron concentrations, as are the nucleotides.

Other ion-pair separations of adenosine compounds have been published. Using a TBA/acetonitrile/water eluent on a Lichrosorb RP8 or RP18 column, Juengling and Kammermeier (6) separated the nucleotides ATP, ADP and AMP from the uracil and guanine nucleotides in 8min in extracts from rat hearts. Grong et al (7) separated H, HR, AMP, ADP and ATP from cat heart extracts in 16min on a Supelcosil LC-18 column with a phosphate/TBA/methanol eluent. Cardiac high-energy

nucleotides were also the interest of Hull-Ryde et al (4). They separated ATP, ADP, H, X, AMP and HR on a Radial Pak-A column with an ammonium phosphate buffer in less than 12min. None of these methods includes all the degradation products of ATP or metabolites of adenosine found in isolated smooth muscle preparations.

The value of simultaneous determination of uv absorbance at two wavelengths and the use of absorbance ratios to help confirm peak purity has been discussed by Simmonds and Harkness (9). The complexity of the chromatograms of smooth muscle extracts (shown in Fig 4) is similar to that often found in the analysis of other samples of biological origin and the ease of obtaining absorbance ratios with the use of dualchannel detection systems is worthy of comment. The method as presented would also be useful for the analysis of adeninebased compounds from incubation medium from smooth muscle experiments, particularly if these included added purine nucleotides. We have previously found that purines in medium from incubations of rat vas deferens are stable if the medium is acidified to about pH 4. However purine-degrading enzymes are released during incubations of guinea-pig ileum and purines are not stable in medium from such incubations without protein precipitation.

It appears that particularly under gradient separation conditions the purity of ion-pair reagents becomes a matter

of crucial importance. This has long been known to be the case for phosphate buffers used in classical HPLC ion-exchange separations (10).

This method is therefore suitable for the analysis of ATP and its uv-absorbing degradation products in isolated smooth muscle preparations as used in experiments in physiology and pharmacology.

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REFERENCES

1. Zakaria, M. and Brown, P.R., J. Chromatogr., 226, 267, 1981.

2. Anderson, F.S. and Murphy, R.C., J. Chromatogr., 121, 251, 1976.

3. Ramos, D.L. and Schoffstall, A.M., J. Chromatogr., 261, 83, 1983.

4. Hull-Ryde, E.A., Cummings, R.G. and Lowe, J.E., J. Chromatogr., 275, 411, 1983.

5. Nissenen, E., Anal. Biochem., 106, 497, 1980.

6. Juengling, E. and Kammermeier, H., Anal. Biochem., 102, 358, 1980.

7. Grong, K., Stangeland, L., Bakken, A.M., Ingebretsen, O.C., Lekven, J. and Farstad, M., Scand. J. Clin. Lab. Invest., 44, 125, 1984.

8. Caronia, J.P., Crowther, J.B. and Hartwick, R.A., J. Liq. Chromatogr., 6, 1673, 1983.

9. Simmonds, R.J. and Harkness, R.A., J. Chromatogr., 226, 369, 1981.

10. Rabel, F.M., J. Chromat. Sci., 18, 46, 1980.